



FACULTY OF VETERINARY MEDICINE
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An exploratory study of *Helicobacter suis* control strategies

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Science is simply common sense at its best.

Thomas Huxley

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Abbreviation key

AGS	human gastric adenocarcinoma cell line
BabA	blood group antigen-binding adhesin
bp	base pair
<i>cagA</i>	cytotoxin-associated gene A
CagA	cytotoxin-associated protein A
<i>cag</i> PAI	cytotoxin-associated gene pathogenicity island
CLSI	Clinical and Laboratory Standards Institute
COX-2	cyclooxygenase 2
Ct	threshold cycle
CT	cholera toxin
CXCL	CXC ligand
DC	dendritic cell
DNA	deoxyribonucleic acid
<i>E. coli</i>	<i>Escherichia coli</i>
ELISA	enzyme-linked immunosorbent assay
FISH	fluorescent <i>in situ</i> hybridization
Fla	flagellin
FldA	<i>H. pylori</i> flavodoxin protein
GGT	γ -glutamyl transpeptidase
<i>gyrA/B</i>	DNA gyrase A/B coding gene
h	hour
<i>H.</i>	<i>Helicobacter</i>
HCl	hydrochloric acid
HE	haematoxylin-eosin
HP-NAP	<i>H. pylori</i> neutrophil-activating protein
HpaA	<i>H. pylori</i> adhesin A
Hsp	heat-shock protein
HtrA	high-temperature requirement A
IFN	interferon
Ig	immunoglobulin
IL	interleukin
kDa	kilo Dalton
Le	Lewis
Lpp20	lipoprotein 20
LPS	lipopolysaccharide
LT	heat-labile enterotoxin of <i>E. coli</i>
MALT	mucosa-associated lymphoid tissue
MHC	major histocompatibility complex
MIC	minimal inhibitory concentration
MLST	multilocus sequencing typing
mRNA	messenger RNA
<i>napA</i>	neutrophil-activating protein coding gene

Abbreviation key

NapA	neutrophil-activating protein
NF- κ B	nuclear factor κ B
NHPH	non- <i>H. pylori Helicobacter</i>
NOD	nucleotide-binding oligomerization domain
OD	optical density
OipA	outer inflammatory protein A
OMP	outer membrane protein
PAGE	polyacrylamide gel electrophoresis
PAMPs	pathogen-associated molecular patterns
PCR	polymerase chain reaction
PgbA/B	plasminogen-binding protein A/B
PRRs	pathogen recognition receptors
qPCR	quantitative real-time PCR
QRDR	quinolone-resistance determining region
r	recombinant
ROS	reactive oxygen species
rRNA	ribosomal RNA
SabA	sialic acid-binding adhesin A
<i>S. aureus</i>	<i>Staphylococcus aureus</i>
SDS	sodium dodecyl sulfate
sLe ^x	sialylated Lewis x
SPF	specific pathogen free
s.s.	sensu stricto
Th	T helper
TNF	tumor necrosis factor
TLR	Toll-like receptor
Treg	regulatory T cell
T4SS	type IV secretion system
UreA/B	urease subunit A/B
VacA	vacuolating cytotoxin
2D	two-dimensional

GENERAL INTRODUCTION

This thesis deals with *Helicobacter suis*, a microorganism that is highly prevalent in pigs and also causes gastric disease in humans.

In the general introduction, first the nomenclature of gastric *Helicobacter* species is briefly discussed, including the background of the name “*Helicobacter (H.) suis*”. Then, prevalence, gastric pathology and clinical implications associated with *H. suis* infections in pigs are described. Additionally, the zoonotic significance of this microorganism is considered. The main aim of this general introduction, however, is to have a closer look at bacterium-host interactions with emphasis on bacterial virulence factors, the host immune response, and the effect of vaccination. Since information with regard to these subjects was very scarce for *H. suis* when we started our PhD studies, other gastric helicobacters and in particular *H. pylori*, are considered here as well. Finally, a brief overview of the literature regarding *in vitro* antimicrobial susceptibility of gastric helicobacters is presented.

1. *Helicobacter suis* infections in pigs and humans

1.1. Nomenclature of gastric non-*Helicobacter pylori* helicobacters

In 1984, Marshall and Warren reported for the first time that gastritis and stomach ulcers in humans can be caused by a Gram-negative bacterium (Marshall and Warren, 1984). This slightly curved bacterium was named “*Helicobacter pylori*” (Goodwin et al., 1989), and is nowadays known as the most prevalent *Helicobacter* species in the human stomach, causing gastritis, peptic ulcer disease, gastric adenocarcinoma and mucosa-associated lymphoid tissue (MALT) lymphoma (Stolte et al., 1993; Kusters et al., 2006). Human gastric biopsy samples, however, harbor bacteria which are morphologically different from *H. pylori* (Heilmann and Borchard, 1991; Debognie et al., 1995). These long spiral-shaped non-*H. pylori* helicobacters (NHPH) are primarily associated with animals but infect humans as well (Haesebrouck et al., 2009). Frequent changes in nomenclature of NHPH colonizing the stomach of humans have caused difficulties in reaching international agreement on this complex group of microorganisms. The initial name, “*Gastrospirillum hominis*” (McNulty et al., 1989) was replaced by “*Helicobacter heilmannii*” (Heilmann and Borchard, 1991). However, subsequent analyses of the 16S ribosomal RNA (rRNA)- and 23S rRNA-encoding genes revealed two different types of “*H. heilmannii*” in the human stomach: “*H. heilmannii*” type 1 and “*H. heilmannii*” type 2. “*H. heilmannii*” type 1 is both morphologically and genetically identical to a bacterium colonizing the stomach of pigs (De

Groote et al., 1999; O'Rourke et al., 2004). “*H. heilmannii*” type 2, however, does not represent a single *Helicobacter* species, but a group of species, all known to colonize the gastric mucosa of cats and dogs: *H. felis*, *H. bizzozeronii*, *H. salomonis*, *H. cynogastricus*, *H. baculiformis* and the recently *in vitro* cultivated *H. heilmannii* sensu stricto (*H. heilmannii* s.s.) (Haesebrouck et al., 2009; 2011).

In 1990, for the first time, large spiral-shaped microorganisms were described in the stomach of pigs with chronic gastritis (Queiroz et al., 1990). These microorganisms were provisionally named “*Gastrospirillum suis*” (Mendes et al., 1990). Based on 16S rRNA sequences, fluorescent *in situ* hybridization (FISH) and electron microscopy, this bacterium was described to be a member of the genus *Helicobacter*. Because at that time this species could not be characterized thoroughly due to the lack of pure *in vitro* isolates, the organism was given the name “*Candidatus Helicobacter suis*” (De Groote et al., 1999). Several research groups tried to cultivate these tightly coiled bacteria present in the stomach of pigs, however all attempts failed (Mendes et al., 1991; Grasso et al., 1996; Cantet et al., 1999; Park et al., 2000; Roosendaal et al., 2000). Only in 2008, the first pure *in vitro* isolate was obtained, which finally led to the description of *H. suis* as a formal species name. Nowadays, the name *H. suis* is worldwide accepted as a new gastric *Helicobacter* taxon corresponding to the above mentioned “*H. heilmannii*” type 1 (Baele et al., 2008).

1.2. *Helicobacter suis* infections in pigs

1.2.1. Prevalence of *H. suis* infections in pigs

H. suis colonizes the stomach of pigs worldwide. Prevalence rates range from 8% to 95%, although most studies report 60% or higher at slaughter age (Barbosa et al., 1995; Grasso et al., 1996; Cantet et al., 1999; Roosendaal et al., 2000; Choi et al., 2001; Hellemans et al., 2007a; Kopta et al., 2010). Though, in younger animals the frequency of *H. suis* infection is much lower. Hellemans et al. (2007a) observed a prevalence of only 2% in suckling piglets, which increased rapidly after weaning with a prevalence of 90% in adult boars and sows. This suggests a possible maternal protection due to the protective effect of milk. For *H. pylori*, passive immunity acquired from immunized dams protects young mice against colonization until weaning (Corthésy-Theulaz et al., 2003). Also, a recent study in infants fed on breast milk showed that *H. pylori*-specific immunoglobulin (Ig) A antibodies may play an important role in preventing acquisition of

infection (Bhuiyan et al., 2010). In porcine milk, next to antibodies, other proteins have been shown to inhibit *H. pylori* colonization in mice by specific *H. pylori*-binding epitopes (Gustafsson et al., 2006). The high prevalence of infection in adult pigs suggests that *H. suis* persists in adult pigs, indicating that the natural immune response against *H. suis* does not lead to its clearance from the stomach. Indeed, it is assumed that once *Helicobacter* infection is established, it persists throughout life (Rhen et al., 2003). The immunological basis of this persistence will be further described in section 3 of this introduction.

1.2.2. Role of *H. suis* in porcine gastric pathology

H. suis infections in pigs have been associated with two main gastric pathologies, being **ulcerations of the non-glandular part** of the stomach and **gastritis**. Numerous studies, in both naturally and experimentally infected pigs showed that *H. suis* infections cause chronic gastritis, which is mainly located in the antrum (Mendes et al., 1991; Queiroz et al., 1996; Park et al., 2000; Hellemans et al., 2007b, De Bruyne et al., 2012).

In pigs, gastric ulcers appear in the non-glandular part of the stomach, called pars oesophagea. This rectangular area around the oesophageal opening is covered with stratified squamous epithelium and does not secrete mucus. Consequently, this epithelial layer offers limited protection against low pH in the lumen and gastric enzymes (Friendship, 2006). Factors impairing blood supply to the mucosa or increasing the exposure of the mucosa of the pars oesophagea to acidic conditions could lead to epithelial insults, followed by hyperkeratosis, erosion and finally ulceration. It is now recognized that gastric ulcer disease in pigs is a multifactorial condition, arising from one or more of the following predisposing factors: particle size of feed (pelleting and fine grinding of feed), the presence of short chain fatty acids in the stomach, concurrent diseases leading to decreased food intake, stress and *H. suis* infections (Hessing et al., 1992; Ayles et al., 1996; Amory et al., 2006; Argenzio and Eisemann, 1996; Robertson et al., 2002; Millet et al., 2012). The exact role of *H. suis* in the development of porcine gastric ulcers is not clear. Some studies indeed reported an association of *H. suis* with gastric ulcers (Barbosa et al., 1995; Queiroz et al., 1996; Roosendaal et al., 2000; Choi et al., 2001; Appino et al., 2006; Krakowka and Ellis, 2006; Proietti et al., 2010), while others did not find this association (Grasso et al., 1996; Melnichouk et al., 1999; Park et al., 2000; Szeredi et al., 2005). These contradictory results could be due to different diagnostic tools used for

demonstrating *H. suis*, differences in sampling, but also differences in virulence between *H. suis* strains.

The pathogenesis of *H. suis*-associated gastric ulcers is still unclear. One research group suggested that *H. suis* can increase the number of endocrine gastrin-producing cells, and decrease the number of somatostatin-producing cells, resulting in increased production of hydrochloric acid (HCl) (Sapierzyński et al., 2007). Additionally, *H. suis* was found in close contact with parietal cells of the fundic region of experimentally *H. suis* infected pigs, which might indicate that the bacterium may have an impact on these HCl-producing cells (Hellemans et al., 2007b). The secretion of excessive amounts of gastric acid may lead to increased contact of the non-glandular part of the stomach with HCl. Consequently, this chronic insult of the pars oesophagea may result in the development of ulcers. Finally, it is also possible that *H. suis* is not the primary cause of ulcers, but instead causes a delay in healing leading to more severe lesions.

Regarding the clinical outcome of *H. suis* infections in pigs, De Bruyne et al. (2012) showed that an experimental *H. suis* infection causes a decreased daily weight gain of approximately 10% in pigs over an average period of 5 weeks, which may be important from an economical point of view. Also, *H. suis* infections might be a source of welfare concern in pig populations worldwide due to pain and stress induced by gastritis and gastric ulcers (Friendship, 2006; De Bruyne et al., 2012).

1.3. Zoonotic significance of *H. suis*

H. pylori is considered to be the major cause of chronic atrophic gastritis, peptic ulceration as well as gastric carcinoma and MALT lymphoma in humans (Marshall and Warren, 1984; Parsonnet et al., 1991; Lehours and Mégraud, 2005; Kusters et al., 2006). However, in 0.1% to 8% of the gastric biopsies, large spiral-shaped NHPH are detected (Stolte et al., 1994; Holck et al., 1997; Yali et al., 1998; Yang et al., 1998; Boyanova et al., 2003; Joo et al., 2007; Yakoob et al., 2012). Infections with these bacteria have been associated with gastritis (Debognie et al., 1995; Stolte et al., 1997; Joo et al., 2007), gastric ulcers (Debognie et al., 1998; Sykora et al., 2003) and gastric cancer (Morgner et al., 1995, 2000) in humans. Compared to *H. pylori*-associated gastritis, gastritis associated with NHPH is mostly less active (Stolte et al., 1997; Joo et al., 2007). Histological examination reveals an inflammation that is generally characterized by

infiltration with lymphocytes and plasma cells (Morgner et al., 1995; Stolte et al., 1997; Sykora et al., 2003).

Interestingly, patients with NHPH gastritis develop more frequently MALT lymphoma than those with *H. pylori* gastritis (1.48% and 0.66%, respectively) (Stolte et al., 1997; 2002). Recently, Flahou et al. (2010) demonstrated that *H. suis* causes MALT lymphoma-like lesions at 8 months post-infection in a Mongolian gerbil model.

Coinfections with both *H. pylori* and NHPH have been described in human patients (De Groote et al., 2005; Van den Bulck et al., 2005a; Ojano et al., 2012; Yakoob et al., 2012). These infections mainly result in aspecific gastro-intestinal symptoms, such as vomiting, epigastric pain and dyspepsia (Ojano et al., 2012; Yakoob et al., 2012). The significance of coinfections in terms of disease development and severity, however, needs to be determined.

H. suis comprises 13.9% to 78.5% of gastric NHPH infections in humans (Trebesius et al., 2001; De Groote et al., 2005; Van den Bulck et al., 2005a). The prevalence of other NHPH in humans (with cats and dogs as natural host) is lower (Trebesius et al., 2001; Van den Bulck et al., 2005a), making *H. suis* the most prevalent NHPH species colonizing the stomach of humans.

Results of multilocus sequencing typing (MLST) revealed that a human *H. suis* strain was closely related to porcine *H. suis* strains, further supporting the hypothesis that *H. suis* infections in humans originate from pigs (Liang et al., 2013). Probably, transmission of *H. suis* from pigs to humans occurs by contact. Indeed, a questionnaire-based study illustrated that individuals having close contact with pigs have a higher risk for contracting “*H. heilmannii*” infections (Meining et al., 1998). Further, a recent study reported that a pig veterinarian suffering from reflux oesophagitis and general dyspeptic symptoms was colonized with *H. suis* (Joosten et al., 2013). MLST analysis of 7 housekeeping genes of *H. suis* revealed a very close relationship with porcine *H. suis* strains, suggesting that the patient contracted the infection through close contact with pigs (Joosten et al., 2013; Liang et al., 2013). Besides contact with pigs, the consumption of raw or undercooked pork might be of importance. Recently, it has been shown that *H. suis* can survive for at least 48 hours (h) in raw minced pork (De Cooman et al., unpublished data). In the same study, in 2 out of 50 commercial minced pork samples, DNA of viable *H. suis* bacteria was detected (De Cooman et al., unpublished data). This might indicate that raw or undercooked pork might occasionally constitute a source of *H. suis* infection for humans.

2. Virulence factors of gastric *Helicobacter* species

Before the start of this thesis, little was known about genes associated with colonization and virulence of *H. suis* or other gastric NHPH. Indeed, most of the research regarding virulence factors of gastric *Helicobacter* species has been done with *H. pylori*. As a consequence, this section is focused on this microorganism.

Figure 1 shows an overview of the main virulence factors of *H. pylori* involved in gastric colonization and pathology. These factors, together with other virulence-associated genes will be described in the following paragraphs.

2.1. Virulence factors and their genes involved in gastric colonization and persistence

A successful gastric colonization of *Helicobacter* depends on many factors including flagella-driven motility in the stomach mucus layer, acid neutralization via urease, oxidative stress resistance and adhesion to gastric epithelial cells mediated by several adhesion factors. A review of the involvement of these factors is given in the following paragraphs.

Acid resistance

Gastric helicobacters have adapted to the acid gastric environment for survival and colonization. The main component of *Helicobacter* acid resistance is the urease enzyme, which converts urea into ammonia and carbon dioxide (Burne and Chen, 2000). The ammonia production generated by this reaction increases the pH. The *H. pylori* urease gene cluster consists of an operon containing *ureA* and *ureB*, encoding the structural subunits of the enzyme, urease subunit A (UreA) and urease subunit B (UreB), respectively, followed by a downstream operon comprising the *ureI* and the urease accessory genes *ureE*, *ureF*, *ureG* and *ureH* (Akada et al., 2000). Urease is a cytoplasmic protein, which is also associated with the surface of viable bacteria after autolysis of surrounding bacteria, so-called “altruistic autolysis” (Dunn and Phadnis, 1998; Marcus and Scott, 2001). Urease activity is essential for *H. pylori* colonization, as urease negative mutants are unable to colonize mice (Eaton et al., 1991; Tsuda et al., 1994; Karita et al., 1995). This activity is regulated by different parameters including the pH, the availability of its substrate, urea, and of its co-factor, nickel. UreI, encoded by the *ureI* gene, is a proton-gated urea channel which controls the intracellular urea concentration and pH and thus assures cytoplasmic urease activity (Scott et al., 2000; Bury-Moné et al., 2001). It has been demonstrated that UreI is

essential for *in vivo* gastric colonization by *H. pylori* (Skouloubris et al., 1998; Mollenhauer-Rektorschek et al., 2002). The *ureEFGH* genes are involved in the Ni^{2+} incorporation into the urease apoenzyme (Cussac et al., 1992). Apart from genes of the urease complex, the hydrogenase accessory proteins HypA (encoded by *hypA*) and HypB (encoded by *hypB*), are important for a normal urease activity. Inactivation of *hypA* and *hypB* reduces *H. pylori* urease activity 40- and 200-fold, respectively (Olson et al., 2001). *H. pylori* urease has also been implicated in damage of gastric epithelial cells through the production of ammonia (Smoot et al., 1990; Sommi et al., 1996). Additionally, the urease protein directly causes cell damage by stimulating macrophage inducible nitric oxide (Gobert et al., 2002). Besides urease, other *H. pylori*-ammonia-producing enzymes facilitating gastric *H. pylori* survival have been described. These include amidase (AmiE), (Skouloubris et al., 1997), formamidase (AmiF) (Skouloubris et al., 2001) and aspartase (AspA), encoded by *amiE*, *amiF* and *aspA*, respectively.

Oxidative stress resistance

Additional to acid neutralization factors, *H. pylori* possesses genes coding for enzymes involved in oxidative stress resistance. Genes encoding *H. pylori* catalase and superoxide dismutase show a high homology to those of intracellular pathogens (Odenbreit et al., 1996; Spiegelhalder et al., 1993), suggesting their role in resistance against killing by polymorphonuclear cells. In addition to catalase and superoxide dismutase, *H. pylori* depends on a family of peroxiredoxins that detoxify organic peroxides. For instance, mutant *H. pylori* cells defective in the alkyl hydroperoxide reductase, AhpC, were more sensitive to *in vitro* oxidative stress conditions, resulting in more DNA fragmentation and coccoid or lysed forms compared to wild-type bacteria (Wang et al., 2004). More recently described are the antioxidant proteins, including the nicotinamide adenine dinucleotide phosphate (NADPH) quinone reductase (MdaB), and an iron sequestering protein, also known as the *H. pylori* neutrophil-activating protein, HP-NAP. The latter has a dual role in inducing and combating oxidative stress resistance. Indeed, Wang et al. (2006) demonstrated in a mouse model that HP-NAP, although able to induce oxygen radicals' release (see paragraph 2.2.), protects *H. pylori* from iron-mediated oxidative DNA damage.

Chemotaxis and motility

Helicobacter species are motile by the presence of flagella, whose filaments consist of two flagellins, flagellin A (FlaA) (encoded by the *flaA* gene) and flagellin B (FlaB) (encoded by the *flaB* gene). Experiments in different animal models with *H. pylori*, *H. felis* and *H. mustelae*, the gastric pathogen of ferrets, illustrated that flagellar motility is essential for gastric *Helicobacter* species to colonize (Eaton et al., 1992; Josenhans et al., 1999; Ottemann and Lowenthal, 2002). Many other proteins are known to be essential in flagellar structure and motility of *Helicobacter*. For instance, mutations in the *flgE* and *fliD* genes, encoding the flagellar hook and flagellar cap proteins, respectively, result in nonmotile bacteria (O'Toole et al., 1994; Kim et al., 1999). *H. pylori* shows taxis (directed motility) towards urea, bicarbonate, cholesterol, arginine and several other amino acids, and moves away from low pH (Lertsethtakarn et al., 2011). In addition to motility, several *in vivo* studies indicated that chemotaxis is required for colonization of *H. pylori* (Foynes et al., 2000; McGee et al., 2005; Terry et al., 2005). Indeed, screening for *in vivo* essential genes in an infection model of *H. pylori* in the Mongolian gerbil yielded an abundance of motility and chemotaxis genes, indicating that *in vivo* both features are prime factors in colonization (Kavermann et al., 2003). For example, the chemotaxis receptor TlpB is required for pH taxis, and *tlpB* mutants are defective for mouse colonization (Croxen et al., 2006).

Adhesins and outer membrane proteins

Many bacterial factors mediate the adhesion of *H. pylori* to the gastric epithelium. *H. pylori* exhibits five families of outer membrane proteins (OMPs): the major OMP family consisting of Hop and Hor proteins, the Hof protein family, the Hom protein family, the iron-regulated OMPs and the efflux pump OMPs. Given the multitude of OMPs and adhesins, it is extremely difficult to test for the contribution of each individual adhesin. Therefore, in this overview only adhesins for which sufficient data about their putative role in the pathogenesis of *Helicobacter* infection are available, will be described.

In 1998, Ilver et al. detected a 78- kilo Dalton (kDa) adhesin on the *H. pylori* outer membrane that recognizes Lewis b (Le^b) ABO blood group antigen on the surface of gastric epithelium, and designated it as “blood group antigen-binding adhesin” (**BabA**) (HopS). Two corresponding *babA* genes encode BabA: *babA1* and *babA2*, but only BabA2 has Le^b binding ability (Yamaoka, 2008). Several studies suggested a correlation between *babA2*⁺ *H. pylori* genotype and increased

risk of developing gastric diseases (Gerhard et al., 1999; Yu et al., 2002; Olfat et al., 2005). In 2002, Mahdavi et al. observed that inactivated *babA1A2* mutants could not bind human ABO/Le^b blood group antigens, but still bound to the gastric epithelium. They identified the sialic acid-binding adhesin (**SabA**) (HopP) as another putative adhesin of *H. pylori*, which adheres to the sialylated Le^x (sLe^x) antigen on the surface of epithelial cells. The SabA is encoded by the *sabA* gene. Besides sLe^x, SabA can also mediate the adherence of *H. pylori* to laminin (Walz et al., 2005). Both, the *babA* and *sabA* expressing profile can be modulated according to alternations in host environment by switching “on” and “off” gene expression (Mahdavi et al., 2002; Solnick et al., 2004; Goodwin et al., 2008). The capacity of rapidly switching “on” and “off” BabA and SabA expression allows a continued adaptation of *H. pylori* binding properties to the glycan profile modifications on host epithelial cells that occur during the inflammation process and contributes to the establishment of an infection.

The outer inflammatory protein A (**OipA**), encoded by the *oipA* gene, is another member of the Hop protein family (HopH) that may serve as an adhesin. It was originally identified as a pro-inflammatory response-inducing protein (Yamaoka et al., 2000). Like for *babA* and *sabA*, the expression of the *oipA* gene can be switched “on” and “off” by a slipped-strand repair mechanism within a cytosine-thymine dinucleotide repeat motif (Saunders et al., 1998; Yamaoka et al., 2000). A functional OipA status is strongly associated with increased interleukin (IL)-8 secretion of epithelial cells *in vitro* and heightens gastric inflammation *in vivo* (Yamaoka et al., 2000; 2002a; b). Aside from OipA, **HomB** is another OMP involved in the host inflammatory response. Indeed, *H. pylori* *homB* knockout mutant strains have reduced ability to induce IL-8 secretion by human gastric epithelial cells, as well as reduced capacity to bind these cells (Oleastro et al., 2008).

The *H. pylori* adhesin A (**HpaA**) is a surface-located lipoprotein that was initially described as a putative N-acetyl-neuraminylactose-binding hemagglutinin (Evans et al., 1988; O’Toole et al., 1995). Several studies have tried to elucidate the function of HpaA in *in vitro* adhesion studies, but the results are not conclusive. For instance, *in vitro* bacterial binding to gastric cell lines was not affected by an inactivated *hpaA* gene (O’Toole et al., 1995; Jones et al., 1997). On the other hand, Carlsohn et al. (2006) described that a *hpaA* mutant *H. pylori* strain is not able to establish colonization in mice, indicating that HpaA is essential for colonization of *H. pylori* in mice.

Other adhesins that have been identified on *H. pylori* are **AlpA** and **AlpB** (Odenbreit et al., 1999; Peck et al., 1999). Inactivation of the respective genes, *alpA* and *alpB* results in decreased adherence to gastric epithelial cells and absence of colonization in a guinea pig model (Odenbreit et al., 1999; 2002; de Jonge et al., 2004). More recently, Snelling and co-authors (2007) found that **HorB** also is a gastric epithelial cell adhesin. They observed that disruption of the *horB* gene reduced *H. pylori* adhesion to gastric epithelial cells by more than twofold. Additionally, insertional mutagenesis in the *horB* gene of a mouse-adapted *H. pylori* strain reduced mouse stomach colonization threefold, indicating its role in gastric colonization.

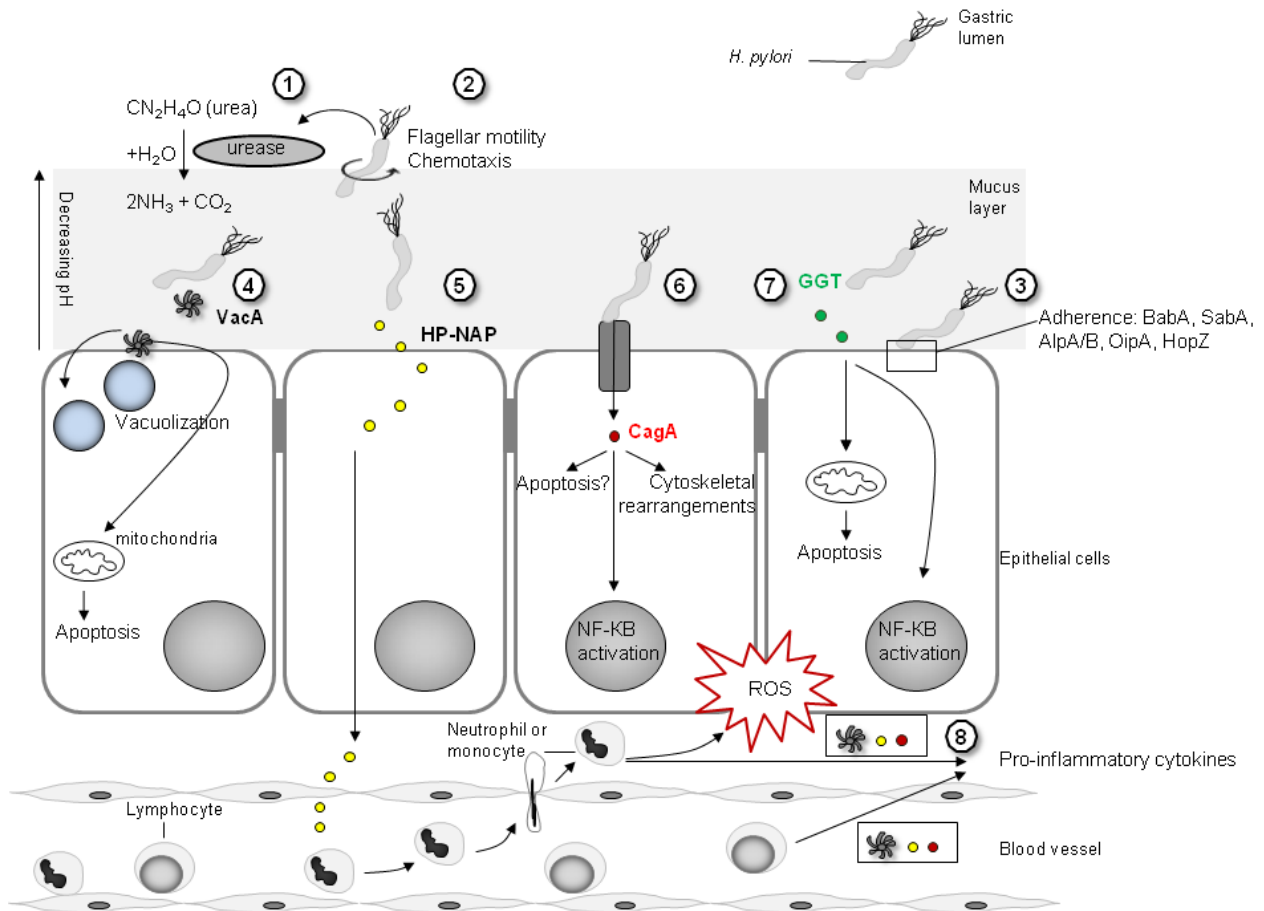


Figure 1. Schematic representation of the stomach colonized with *H. pylori*, showing the main *H. pylori* virulence factors involved in colonization and gastric pathology. (1) The *H. pylori* urease allows survival in the acid environment by hydrolyzing urea into ammonia and carbon dioxide. The ammonia molecules buffer cytosolic and periplasmic pH as well as the surface layer around the bacterium. Flagella propel the bacterium into the mucus layer, and this in a directed way, so-called chemotaxis. For example, *H. pylori* moves away from low pH (2). Once *H. pylori* reaches the apical domain of gastric epithelial cells, it attaches to the cells using specialized adhesins (3). BabA and SabA adhere to blood group antigen receptors, whereas the receptors of OipA, AlpA/B and HopZ are not known. The secreted vacuolating cytotoxin, **VacA**, induces the formation of large vacuoles in epithelial cells and mitochondrial-mediated apoptosis (4). The neutrophil-activating protein, **HP-NAP** (yellow sphere), after crossing the epithelial lining and endothelium, recruits neutrophils and monocytes, which extravasate and cause tissue damage by releasing reactive oxygen species (ROS). *H. pylori* can inject a cytotoxin-associated protein, **CagA** (red sphere), into host cells by a specialized type IV secretion system, encoded by the *cag* PAI (6). Injected CagA causes alterations of the cytoskeleton, can induce apoptosis *in vitro* and signals the nucleus to induce nuclear factor (NF)- κ B. The *H. pylori* γ -glutamyl transpeptidase, **GGT** (green sphere), is involved in mitochondrial-mediated apoptosis and induces NF- κ B. Finally, both VacA, CagA and HP-NAP can induce production of pro-inflammatory cytokines by lymphocytes and/or polymorphonuclear cells (8).

2.2. Virulence factors and their genes involved in induction of gastric lesions

Cytotoxin-associated gene pathogenicity island

In 1990, a strain-specific *H. pylori* gene, cytotoxin-associated gene A (*cagA*), was identified (Cover et al., 1990), which is now recognized as a major virulence factor of *H. pylori*. No homologs are known for *cagA* in other *Helicobacter* species. *cagA* is a marker for the *H. pylori* cytotoxin-associated gene pathogenicity island (*cag* PAI), which is present in virulent strains but missing in avirulent *H. pylori* isolates (Akopyants et al., 1998). The *cag* PAI consists of about 30 genes (Akopyants et al., 1998) and encodes a type IV secretion system (T4SS) which mediates the injection of cytotoxin-associated effector protein (CagA) into epithelial cells, both *in vitro* (Segal et al., 1999; Odenbreit et al., 2000) and *in vivo* (Yamazaki et al., 2003). In contrast with other bacterial T4SS, the *cag* PAI exhibits specific *cag* genes, of which only a few encode proteins with clear sequence similarities to known T4SS VirB and VirD proteins (Fischer et al., 2010). Once within the cytoplasm of the eukaryotic cell, CagA protein is phosphorylated at tyrosine residues at its five amino-acid Glu-Pro-Ile-Tyr-Ala (EPIYA) repeat region (Backert et al., 2001) by the host cell Src kinases (Stein et al., 2002). Phosphorylated CagA interacts with the Src homology 2 (SH2) domains of the tyrosine phosphatase SHP-2 (Higashi et al., 2002), which affects migration, spreading and adhesion of epithelial cells (Yamazaki et al., 2003). This phenomenon of cytoskeleton rearrangements, inducing dramatic elongation and spreading of epithelial cells has been called “hummingbird” phenotype and depends on the phosphorylation of CagA (Segal et al., 1999). CagA also interacts with Grb2, which results in activation of the Ras→Mek→Erk pathway, leading to the activation of pro-inflammatory nuclear factor (NF)-κB (Brandt et al., 2005), cytoskeletal rearrangements and proliferation of cells *in vitro* (Mimuro et al., 2002). Conflicting reports exist about the effect of CagA on apoptosis in human epithelial cells. One study described that CagA promotes the expression of anti-apoptotic myeloid cell leukemia sequence 1 and prosurvival factor phospho-Erk *in vivo* (Mimuro et al., 2007). In contrast, Tsutsumi et al. (2003) demonstrated that CagA increases apoptosis of human gastric adenocarcinoma cell line (AGS) *in vitro*. Human infections with *cagA*-positive *H. pylori* strains mainly show overexpression of pro-apoptotic proteins. However, to a lesser extent anti-apoptotic proteins are also overexpressed (Cabral et al., 2006). It has been suggested that the anti-apoptotic effects of CagA may aid persistence by maintaining the turnover of the epithelial cells to which *H. pylori* bacteria are attached (Mimuro et al., 2007). In any case, the *cagA*⁺ status of *H. pylori*

strains has been identified as possible virulence marker for disease outcome as it is associated with increased risk for peptic ulcer disease (Nomura et al., 2002) and gastric cancer (Blaser et al., 1995; Wen and Moss, 2009).

Vacuolating cytotoxin

Another major virulence factor of *H. pylori*, is the vacuolating cytotoxin (VacA). This protein is delivered to the host cell's cytoplasm by releasing outer membrane vesicles. The first documented effect of VacA was its ability to induce gastric epithelial cell vacuolization (Cover and Blaser, 1992). The vacuolization is dependent on the formation of membrane channels by the toxin as it is completely abrogated by chloride channel inhibitor 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB) (Szabò et al., 1999). VacA also causes alterations in mitochondria, such as reduction of the mitochondrial transmembrane potential and release of the mitochondrial cytochrome c, which could lead to apoptosis or impaired cell cycle of host cells (Kimura et al., 1999; Willhite and Blanke, 2004). Indeed, several studies showed that *in vitro* exposure of AGS cells to VacA leads to apoptosis of these cells (Kuck et al., 2001; Cover et al., 2003). The toxicity of VacA is dependent on variations of the signal (s) and middle (m) regions of its encoding gene, *vacA*. The s1/m1 allele has been shown to be the most virulent form of the *vacA* gene whereas the s2/m2 genotype did not show any vacuolating activity (Atherton et al., 1995). Interestingly, the s1/m1 type of *vacA* is clearly linked with *cagA*⁺ genotype, indicating that *vacA* alone could not be considered as a virulence marker for disease outcome. Yamaoka et al. (1999) indeed observed that the *vacA* type alone is a poor predictive marker for disease severity.

VacA is also able to execute either pro-inflammatory or immune suppressive effects. On the one hand, VacA stimulates the expression of cyclooxygenase 2 (COX-2) in neutrophils and macrophages and activates lymphocytes and mast cells to produce pro-inflammatory cytokines (Supajatura et al., 2002; de Bernard et al., 2005). On the other hand, VacA is able to inhibit the activation of nuclear factor of activated T cells (NFAT), and consequently the production of IL-2, required for T cell proliferation (Boncristiano et al., 2003; Gebert et al., 2003).

In *H. felis*, *H. bizzozeronii*, *H. heilmannii* s.s. and *H. mustelae*, no *vacA* orthologs has been found yet (O'Toole et al., 2010; Arnold et al., 2011; Schott et al., 2011; Smet et al., 2013).

Neutrophil-activating protein

Another *H. pylori* virulence factor with pro-inflammatory capacities is HP-NAP. HP-NAP, encoded by the *napA* gene, was first identified to stimulate the production of toxic reactive oxygen species (ROS) in neutrophils and promote adhesion of neutrophils to endothelial cells (Evans et al., 1995). Purified recombinant HP-NAP was found to be chemotactic for human neutrophils and monocytes *in vitro* (Satin et al., 2000). Moreover, using intravital microscopy, it has been demonstrated that in rats HP-NAP is able to cross the endothelia efficiently and to promote rapid neutrophil adhesion *in vivo* (Polenghi et al., 2007). After crossing the epithelial monolayer, HP-NAP is able to activate the underlying mast cells (Montemurro et al., 2002), monocytes (Montemurro et al., 2001; Amedei et al., 2006), and neutrophils (Polenghi et al., 2007) to release pro-inflammatory cytokines. It has been suggested that the presence of a large number of positively charged residues on the surface of HP-NAP might account for its unique ability in activating human leucocytes (Zanotti et al., 2002). A recent study added evidence that HP-NAP further contributes to inflammation by increasing the lifespan of monocytes and neutrophils (Cappon et al., 2010). This study showed that *in vitro* exposure of monocytes to HP-NAP induced anti-apoptotic proteins, and reported that HP-NAP promotes survival of neutrophils in a monocyte-dependent manner (Cappon et al., 2010).

HP-NAP is a Toll-like receptor (TLR)2 agonist with immune modulatory capacities, able to induce the expression of IL-12 and IL-23 by human neutrophils and monocytes. In fact, HP-NAP has the potential to shift antigen-specific T cell responses from a predominant T helper (Th)2 to a polarized Th1 cytotoxic phenotype, characterized by high levels of interferon (IFN)- γ and tumor necrosis factor (TNF)- α production (Amedei et al., 2006; D'Elios et al., 2007). Homologs of the HP-NAP coding gene, *napA*, have also been found in the genome of *H. felis*, *H. bizzozeronii*, *H. mustelae* and *H. heilmannii* s.s. (O'Toole et al., 2010; Arnold et al., 2011; Schott et al., 2011; Smet et al., 2013).

γ -glutamyl transpeptidase

In 1999, Chevalier and co-authors identified a catalytically active enzyme, γ -glutamyl transpeptidase (GGT), as essential for colonization of mice. GGT activity is found in all gastric *Helicobacter* species and is constitutively expressed *in vivo* and *in vitro* (Haesebrouck et al., 2009; Wachino et al., 2010). Additionally, Rossi et al., (2012) recently showed evidence that

GGT has a conserved function in the *Helicobacter* genus. The role of GGT as virulence factor of gastric *Helicobacter* species has only been investigated for *H. pylori* and recently for *H. suis*. In *H. pylori*, GGT induces apoptosis of AGS cells in a dose-dependent manner (Shibayama et al., 2003). Later, the authors showed that *H. pylori* GGT very efficiently utilizes both glutamine and glutathione from epithelial cells as a source of glutamate. As glutamine and glutathione are important nutrients for maintenance of healthy gastrointestinal tissue, their depletion by the GGT enzyme was hypothesized to account for the damaging of gastric epithelial cells (Shibayama et al., 2007). A later study confirmed that *H. pylori* GGT causes apoptosis of AGS cells and demonstrated that the *H. pylori* GGT-induced apoptosis of AGS cells occurs via a mitochondria-mediated pathway (Kim et al., 2007). More recent work of the same research group showed that recombinant *H. pylori* GGT inhibits growth of AGS cells by inducing cell cycle arrest in the G1-S transition phase (Kim et al., 2010). Aside from apoptosis, *H. pylori* GGT also upregulates expression of COX-2 and epidermal growth factor (EGF)-related peptides in human gastric cells, suggesting a possible role in *H. pylori*-associated gastric carcinogenesis (Busiello et al., 2004). A better understanding of the clinical importance of GGT has been recently obtained from a study of Gong et al. (2010). They observed significantly higher GGT activity in *H. pylori* isolates obtained from patients with peptic ulcer disease than isolates from patients with nonulcer dyspepsia. In the same study, purified native *H. pylori* GGT caused an increase of oxidative DNA damage and generated H₂O₂ in primary gastric epithelial and AGS cells, resulting in the activation of NF- κ B, that in turn lead to upregulation of pro-inflammatory IL-8.

The role of *H. suis* GGT in gastric pathology has recently been examined by Flahou et al. (2011). Incubation of AGS cells with purified native or recombinant *H. suis* GGT showed that also the *H. suis* GGT induces apoptosis of AGS cells (Flahou et al., 2011). Additionally, they demonstrated that the induction of epithelial cell death caused by *H. suis* and *H. pylori* GGT is mainly glutathione degradation-dependent. The degradation products, including cysteinyl glycine, induce an increase of H₂O₂, which in turns results in oxidative cell damage (Flahou et al., 2011).

H. pylori, but also the enterohepatic *H. bilis* GGT has immune modulatory capacities, playing a role in suppression of the host immune response (Gerhard et al., 2005; Schmees et al., 2007; Rossi et al., 2012). This will be further described in paragraph 3.3.

Other virulence factors

Besides the extensively studied major *H. pylori* virulence genes, *cagA*, *vacA*, and the more recently discovered virulence factors GGT and HP-NAP, *H. pylori* harbors other virulence-associated candidates. The role and association with pathology of these candidates, including the duodenal ulcer promoting gene A (*dupA*), *H. pylori* flavodoxin protein (FldA), *iceA* (“induced by contact with epithelium”), the high temperature requirement A (HtrA) and plasminogen-binding proteins A and B (PgbA and PgbB) are described in Table 1. To our knowledge, *dupA* and *iceA* have only been found in the *H. pylori* genome, whereas *htrA*, *fldA* and *pgbA/B* were also found in the genome of other gastric *Helicobacter* species (O’Toole et al., 2010; Arnold et al., 2011; Schott et al., 2011; Smet et al., 2013).

Table 1. *H. pylori* less documented virulence-associated factors and their association with *H. pylori*-related disease

Protein/gene	Predicted role	Association with <i>H. pylori</i> -related disease/effects	References
DupA/ <i>dupA</i>	<i>dupA</i> encodes for a VirB4 ATPase homolog. There are two alleles: <i>dupA1</i> and its truncated version <i>dupA2</i>	<ul style="list-style-type: none"> • Gastric and duodenal ulcers • Gastric cancer, but this association is not universal • <i>dupA1</i> increases IL-12p40, IL-12p70 and IL-23 expression by mononuclear cells 	Lu et al., 2005; Hussein, 2010; Hussein et al., 2010
IceA/ <i>iceA</i>	<i>iceA1</i> allele encodes a CATG-recognizing restriction endonuclease	<ul style="list-style-type: none"> • IceA1: associated with peptic ulcers, but this association is not universal 	Peek et al., 1998; Yamaoka et al., 1999; Donahue et al., 2000; Kidd et al., 2001; Xu et al., 2002
FldA/ <i>fldA</i>	Electron acceptor of the pyruvate oxidoreductase enzyme complex	<ul style="list-style-type: none"> • Antibodies against FldA are associated with gastric MALT lymphoma in humans 	Chang et al., 1999
HtrA/ <i>htrA</i>	Cleaving ectodomain of E-cadherin, a mammalian cell-adhesion protein	<ul style="list-style-type: none"> • <i>In vivo</i> and <i>in vitro</i> disruption of gastric epithelial barrier junctions, facilitating intercellular entry of <i>H. pylori</i> 	Hoy et al., 2010; Löwer et al., 2011
PgbA, PgbB/ <i>pgbA</i> , <i>pgbB</i>	Binds host plasminogen	<ul style="list-style-type: none"> • Could enhance tissue damage by proteolysis • May obstruct natural healing process of gastric ulcers 	Ljung et al., 2000; Jönssen et al., 2004

dupA: duodenal ulcer promoting gene, ***iceA***: “induced by contact with epithelium” coding gene, ***fldA***: *H. pylori* flavodoxin coding gene, **MALT**: mucosa-associated lymphoid tissue, ***htrA***: “high-temperature requirement A” coding gene, ***pgbA*** and ***pgbB***: plasminogen-binding protein coding gene A and B.

3. Host immune response against gastric *Helicobacter* infections

In contrast to the scarce literature available on host immune responses against NHPH, numerous studies evaluated the host immune response against *H. pylori*. This section is therefore focused on *H. pylori*. Where possible, recent insights in *H. suis*-induced immune responses will also be discussed.

In humans, *H. pylori* often elicits a distinct host immune response, which is however inefficient for bacterial clearance without treatment (Blaser and Atherton, 2004). The underlying mechanism for persistence of *H. pylori* in the human stomach remains incompletely understood. Insights in the complex immunobiology of chronic *H. pylori* infections are helpful for the development of an effective vaccine.

The following section reviews how *H. pylori* establishes and maintains persistent infection, mainly in mouse models, complemented with results from *in vitro* experiments and studies in *H. pylori*-infected humans. Both innate and acquired host immune responses to *H. pylori* are described, followed by the role of *H. pylori* virulence factors in evasion of the host immune response.

3.1. Innate immune response to *H. pylori*

Since *H. pylori* is a predominantly extracellular bacterium (Dubois and Borén, 2007), innate immune cells, including dendritic cells (DCs) and macrophages as well as gastric epithelial cells, form a first barrier to infection. They detect invading pathogens via conserved microbial structures, the so-called pathogen-associated molecular patterns (PAMPs). PAMPs are recognized by pattern recognition receptors (PRRs) located either on cytoplasmic and endosomal membranes or in the cytosol of host cells. The surface-expressed PRRs, Toll-like receptors (TLRs), play an important role in mucosal host defense against *H. pylori*. In particular, TLR4, -5 and -9 have been detected on gastric epithelial cells of humans suffering from *H. pylori*-gastritis as well as in patients with a non-inflamed gastric mucosa (Schmausser et al., 2004). Interestingly, *H. pylori* differs from other gastrointestinal pathogens in that it has evolved to largely avoid recognition of its PAMPs. First, although lipopolysaccharide (LPS) is the classical bacterial ligand for TLR4, *H. pylori*-derived LPS has a weak affinity for this receptor and mainly acts via TLR2 (Bäckhed et al., 2003; Ferrero, 2005; Cullen et al., 2011). Second, *Helicobacter* flagellin has been shown to evade the TLR5-mediated innate immunity (Gewirtz et al., 2004; Lee et al.,

2003). This is possibly due to the ability of *H. pylori* to modify its flagellar TLR recognition site, rendering the flagellin protein unable to activate TLR5 (Andersen-Nissen et al., 2005). Besides TLRs, another family of PRRs, the cytosolic nucleotide-binding oligomerization domain (NOD) proteins, appear to play a central role in mediating innate immunity against *H. pylori*. NOD1 is involved in the intracellular recognition of bacterial mucopeptides derived from bacterial peptidoglycan. Viala et al. (2004) found that *H. pylori* activates NOD1 in gastric epithelial cells by introducing its peptidoglycan into the cytoplasm of epithelial cells through its T4SS. This *H. pylori*-mediated NOD1 signaling resulted in the activation of NF- κ B and a subsequent production of pro-inflammatory cytokines, including CXCL-8 (IL-8), CXCL-2 (a murine IL-8 homolog) (Viala et al., 2004) and type 1 IFN (Watanabe et al., 2011). *H. pylori* is also able to avoid detection by NOD1. Indeed, Chaput et al. (2006) demonstrated that transformation of *H. pylori* to coccoid forms, which are still able to infect (Wang et al., 1997), results in modification of cell wall peptidoglycan, which in turns allows coccoid forms to escape detection by NOD1.

The production of chemokines by gastric epithelial cells after *H. pylori* infection facilitates a second stage in the innate immune response: the recruitment of innate immune cells such as neutrophils, macrophages and DCs. Phagocytosis is an important anti-bacterial innate immune mechanism, and a substantial phagocytic cell infiltrate (macrophages and neutrophils) can be found in *H. pylori*-infected gastric mucosa. However, *H. pylori* partially evades phagocyte-mediated killing. Indeed, a large proportion of bacteria seems to survive inside phagosomes which may provide a protected intracellular niche contributing to the persistence of infection (Allen et al., 2000; Allen, 2001). Additionally, *H. pylori* induces the generation and extracellular release of ROS, but is capable of surviving this response through neutralization by its catalase activity (Ramarao et al., 2000).

In response to *H. pylori* antigens, macrophages and DCs are stimulated leading to recruitment, differentiation and activation of CD4⁺ Th cells, which organize the adaptive immune response (Wilson and Crabtree, 2007).

3.2. Acquired immune response to *H. pylori*

The predominant T cell response in the gastric mucosa of *H. pylori*-infected humans includes an IFN- γ -producing CD4⁺ T cell phenotype (D'Elios et al., 1997; Bamford et al., 1998). This **Th1 cell response** is associated with reduced bacterial colonization density in humans (Holck et al., 2003) and in mouse models (Lucas et al., 2001; Stoicov et al., 2004) (Figure 2). Additionally, the development of *H. pylori*-induced gastric pathology largely depends on Th1 cell-mediated responses and Th1 cytokines (Mohammadi et al., 1996; Smythies et al., 2000; Sommer et al., 2001). This has been confirmed in animal models, using for instance IL-4^{-/-} and IFN- γ ^{-/-} mice (Smythies et al., 2000), IFN- γ neutralization in a *H. felis* mouse model (Mohammadi et al., 1996), and mice deficient in Th1 cell development (Sommer et al., 2001). A number of *H. pylori* virulence factors have been reported to promote Th1 responses. For example, HP-NAP promotes the expansion of IFN- γ -producing cells (i.e. the Th1 phenotype) in antigen-stimulated cell cultures, while decreasing the number of IL-4-secreting cells (i.e. the Th2 phenotype) (Amedei et al., 2006). In addition, the *cag* PAI is associated with increased IL-12 and IL-18 responses (Wang et al., 2007; Yamauchi et al., 2008; Takeshima et al., 2009), and OipA stimulates IL-18, which in turns results in a Th1 response (Yamauchi et al., 2008).

In addition to Th1 cells, a **Th2 phenotype** has been observed in *H. pylori*-infected gastric mucosa (Serrano et al., 2007; Robinson and Atherton, 2010). In contrast to Th1 cells, the Th2 cell response suppresses *H. pylori*-induced inflammation and is associated with increased *H. pylori* colonization (Fox et al., 2000; Smythies et al., 2000) (Figure 2).

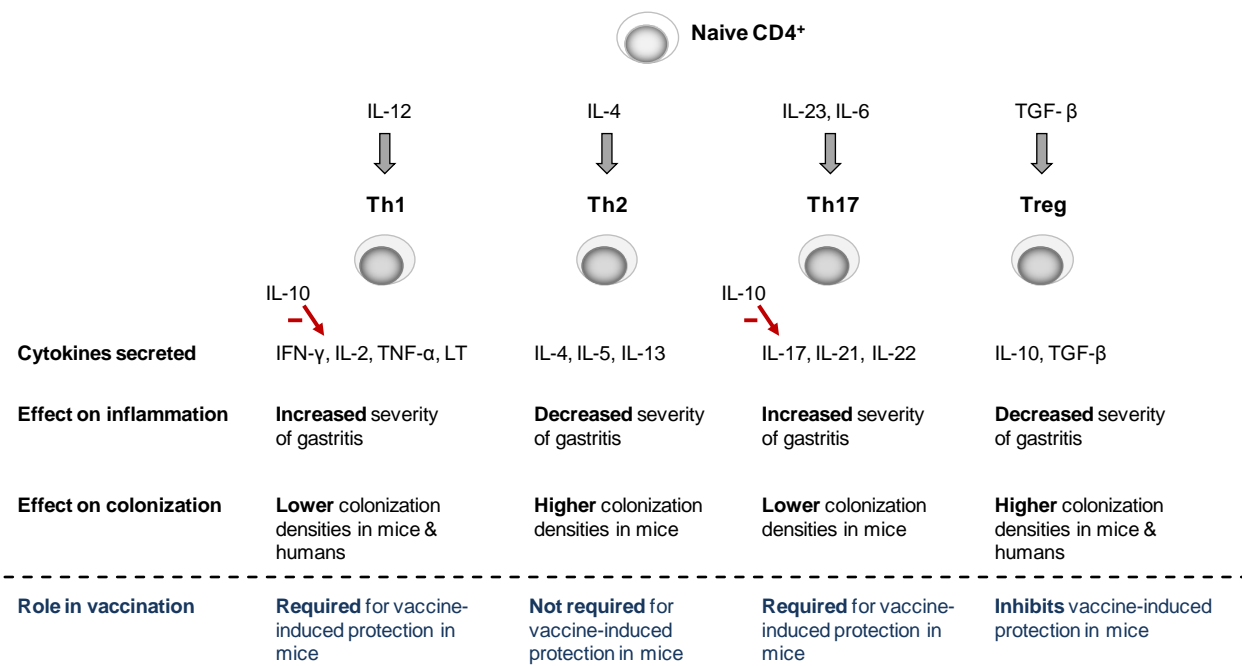


Figure 2. Effect of CD4⁺ cell subsets on gastric *H. pylori* colonization in mice and/or humans, pathological outcome of infection and vaccination in mice. In addition, the innate factors that influence their differentiation from naive T cells and the cytokines they secrete are shown. IL-10 down-regulates both Th1 and Th17 responses. Th: T helper cell, Treg: regulatory T cell. IFN-γ: interferon γ, IL: interleukin, LT: lymphotoxin, TNF-α: tumor necrosis factor α, TGF-β: transforming growth factor β.

IL-17-secreting T cells, **Th17 cells**, have also been identified in the stomach of *H. pylori* infected humans and play a role in inducing IL-8 secretion by gastric epithelial cells and recruitment of neutrophils to the site of infection (Luzza et al., 2000; Mizuno et al., 2005). Numerous studies demonstrated that IL-17 is a pro-inflammatory cytokine and that the Th17/IL-17 pathway plays an important role in the inflammatory response to *H. pylori* infection (Shiomi et al., 2008; Shi et al., 2010; Hitzler et al., 2012; Ragavan and Quiding-Järbrink, 2012). For instance, Shiomi et al. (2008) observed that IL-17^{-/-} mice showed decreased gastric inflammation and neutrophil infiltration compared to wild-type mice. This has been confirmed in another study in which gastric inflammation was decreased when IL-17 activity was blocked *in vivo* (Shi et al., 2010). Further, mice infected with *H. pylori* showed a mixed Th17/Th1 response, with the Th17/IL-17 pathway preceding and modulating the Th1 response. Recently, Hitzler et al. (2012) demonstrated that *H. pylori*-associated gastritis is reduced in the absence of Th17-polarizing cytokine IL-23.

The study of Serelli-Lee et al. (2012) strengthens the importance of IL-17 in *H. pylori*-induced chronic inflammation. They found that persistent *H. pylori*-specific Th17 responses, associated with elevated gastric IL-1 β , occurred in humans with past *H. pylori* infection. This suggests that the persistent inflammation, induced by IL-17 and IL-1 β , might contribute to the development of gastric cancer. Finally, most studies reveal a negative correlation between a Th17 response and *H. pylori* colonization (Scott Algood et al., 2009; Kao et al., 2010), although some authors suggest the opposite (Shi et al., 2010).

H. pylori uses a variety of mechanisms to inhibit the T cell response and to persist in the gastric mucosa. **Regulatory T cells** (Tregs) are mainly CD4⁺ T cells expressing high levels of CD25, and inhibit the effector function of other immune cells by producing anti-inflammatory cytokines such as IL-10 and transforming growth factor (TGF)- β (Raghavan and Quiding-Järbrink, 2012). Many studies reported that *H. pylori* may induce a Treg response, leading to *H. pylori* persistence (Lundgren et al., 2003; Raghavan et al., 2003; 2004; Rad et al., 2006; Nedrud et al., 2012). Indeed, DCs exposed *in vitro* to *H. pylori* antigens have been shown to skew T cell response from Th17/Th1 towards Treg differentiation, which reduces effector responses (IFN- γ and IL-17) and in turn clearance of the infection (Kao et al., 2010). Robinson et al. (2008) suggested that low numbers of IL-10-secreting Tregs results in an increased Th1 response, leading to more severe gastritis. This has been confirmed by Harris et al. (2008), who showed that gastric Treg responses down-regulate the inflammation and ulceration by *H. pylori* in children. The influence of CD4⁺ T cell subsets upon outcome of *H. pylori* infection, complemented with their role in *H. pylori* vaccine-mediated immunity is summarized in Figure 2.

In summary, a *H. pylori* infection mainly leads to specific Th17/Th1 immune responses. Additionally, the induction of IL-10-secreting Tregs could result in evasion of the host immune system and control of immunopathology caused by Th1 and Th17 responses (Robinson and Atherton, 2010).

A *H. pylori* infection stimulates the production of mucosal and systemic anti- *H. pylori* IgA and IgG antibodies (Ferrero et al., 1998; Meining et al., 2002), but the effect of antibody responses on bacterial colonization remains controversial. One study reported that the intragastric administration of specific monoclonal IgA mediated protection against *H. felis* infection in mice (Czinn et al., 1993). In contrast, others showed that specific IgA and IgG in mice actually promote bacterial colonization and impair the host resistance against *H. pylori* infection (Akhiani et al., 2004; 2005). The role of antibodies in limiting *H. pylori* infection is further discussed in section 4.

3.3. Role of *H. pylori* virulence factors in host immune evasion

H. pylori has virulence factors that allow the bacterium to suppress CD4⁺ T cell-mediated immunity. VacA has been shown to inhibit T cell proliferation by interfering with the T cell receptor/IL-2 signaling pathway at the level of calcineurin, i.e. the Ca²⁺-calmodulin-dependent phosphatase (Boncristiano et al., 2003; Gebert et al., 2003). Additionally, one study demonstrated that VacA may interrupt phagosome maturation in macrophages, allowing *H. pylori* strains expressing VacA to evade the innate host immune response (Zheng and Jones, 2003).

Gerhard and co-workers (2005) showed that a *H. pylori* secreted low-molecular weight protein, distinct from VacA, arrests antigen-activated T cells in the G1 phase of the cell cycle by interfering with G1 cyclin-dependent kinase activity. The authors demonstrated later through a biochemical approach that the secreted γ -glutamyl transpeptidase of *H. pylori* (GGT) is the responsible factor for inhibition of T cell proliferation (Schmees et al., 2007). Indeed, mutagenesis of GGT abrogated the inhibitory effect of the bacteria and recombinantly expressed GGT showed anti-proliferative activity (Schmees et al., 2007).

3.4. Host immune response against *H. suis*

Little is known about the immune response against *H. suis* infections, because isolation of *H. suis* and *in vitro* cultivation of this bacterium have only been possible since 2008.

Some studies described the immune response after inoculation of rodents with gastric tissue suspensions containing *H. suis*. One study observed that an infection with “*H. heilmannii*” type 1, originating from a pig stomach and which in fact is *H. suis*, leads to increased messenger (m)RNA expression of IFN- γ and IL-10 in the mouse stomach, indicating that both a Th1 response and Th2 response are associated with “*H. heilmannii*” type 1 infection (Park et al., 2008). Cinque et al. (2006) observed that “*H. heilmannii*” type 1-infected IFN- γ ^{-/-} mice did not show gastric follicular gastritis, whereas wild-type and IL4-deficient infected mice developed this pathology. The authors concluded that IFN- γ plays an essential role in the pathogenesis of gastric lymphoid follicles formation in “*H. heilmannii*” type 1-infected mice. In two other studies, mRNA expression levels of IFN- γ were upregulated in the gastric mucosa of C57BL/6 mice at 3 to 6 months after inoculation with a gastric tissue homogenate containing *H. suis*, indicating a Th1 response (Nobutani et al., 2010; Mimura et al., 2011).

On the other hand, Flahou et al. (2012) observed in the gastric mucosa of both BALB/c and C57BL/6 mice, experimentally infected with *H. suis* a mild upregulation of the Th2 signature cytokine, IL-4, but no upregulation of IFN- γ mRNA expression. It has to be emphasized that only in the study of Flahou et al. (2012) pure cultures of *H. suis* were used, whereas in all other studies animals were inoculated with homogenates of gastric tissue not only containing *H. suis*, but also other bacteria. This might explain these discrepancies. Differences in *H. suis* strains and animal models might also play a role.

While literature describes a Th1 or Th2 response, all studies agree on the presence of a Th17 response in *H. suis*-infected rodent models. Gastric expression levels of both IL-17 and IL-6 (promoting Th17 response) mRNA were negatively correlated with *H. suis* colonization in mice (Flahou et al., 2012) and IL-17 mRNA was also upregulated in Mongolian gerbils infected with *H. suis* (unpublished data). Despite a fully functional Th17 response, with subsequent infiltration of neutrophils, *H. suis* colonization has been shown to persist in mice and gerbils.

In summary, in the stomach of mice inoculated with pure cultures of *H. suis*, a predominant Th17 response is induced, accompanied by a less pronounced Th2 response (Flahou et al., 2012). This contrasts with the Th17/Th1 response and the absence of Th2 response induced by *H. pylori* infection in these rodent models (Robinson and Atherton, 2010). These discrepancies could possibly explain the different pathologies induced by both *Helicobacter* species, i.e. more lymphoid lesions in case of *H. suis*, and predominant metaplastic/dysplastic changes in case of *H. pylori* infections (Wiedemann et al., 2009; Flahou et al., 2010).

4. Prophylactic and therapeutic control of gastric *Helicobacter* infections

Human gastric infections with *H. pylori* occur in one-half of the world's population. Similarly in pigs, infections with *H. suis* show a very high prevalence worldwide (Haesebrouck et al., 2009). Although combined antimicrobial and anti-acid therapy can successfully eradicate *H. pylori* in most patients, increasing antimicrobial resistance in the bacterium remains a serious problem (Selgrad and Malfertheiner, 2008). Additionally and from an epidemiological point-of-view, *Helicobacter* infections are so widespread that antimicrobial treatment is an impractical approach for controlling *Helicobacter* infections. As described previously in section 3, gastric *Helicobacter* infections are not readily cleared by the host inflammatory and immune responses, and may persist for life. Vaccination approaches eliciting a protective immune response have therefore been studied extensively for *H. pylori* since the early nineties. This section will mainly focus on vaccination experiments against gastric *Helicobacter* infections. Additionally, as part of a therapeutic perspective, the *in vitro* antimicrobial susceptibility of gastric *Helicobacter* species will be described.

4.1. Vaccination against gastric *Helicobacter* infections

In this paragraph, a brief overview of approaches to discover candidate protective antigens will be given. Next, the *in vivo* validation of candidate protective antigens in vaccination experiments against gastric *Helicobacter* infections will be discussed. Finally, the aspects of the immune response induced by vaccination and their link with protective immunity will be given.

4.1.1. Selection of candidate protective antigens for subunit vaccines

For 20 years, a number of researchers have been pursuing the objective of developing vaccines against *H. pylori*. Initial, but also recent attempts, have made use of inactivated whole-cell vaccines. However, the use of whole-cell preparations for vaccination implies potential drawbacks. First, whole-cell lysates could contain LPS fractions, which may induce undesirable side-effects (Moran et al., 2002). Second, whole-cell vaccines consist of a complex mixture of many antigens, including both protective antigens, and antigens suppressing protection (Haesebrouck et al., 2004). Therefore, many laboratories began investigating specific purified or recombinant proteins, with less potential for side-effects, as candidates for subunit vaccination in

mice. The discovery of candidate protective antigens could be based on different approaches, including the pregenomic approach, the genomic approach, and the proteomic approach.

Most *Helicobacter* antigens, considered as potential vaccine candidates, were identified in the pregenomic era (Del Giudice et al., 2001). This approach is based on the involvement of the protein in virulence and/or colonization of *Helicobacter* and/or on its abundance. In this way, the urease enzyme, CagA, VacA and others have been selected as candidates for subunit vaccines.

Since the first description of the *H. pylori* genome (Tomb et al., 1997), the genomic based approach for selecting potential antigens has gained interest. The complete genome sequencing of a bacterium represents a large reservoir of genes encoding potential antigens that can be selected and tested as vaccine candidates. Potential vaccine candidates, including membrane- or surface-associated proteins, can be identified in a reverse manner, based on *in silico* predictions. This approach has been used for many bacterial species and has been termed “reverse vaccinology” (Rappuoli, 2001; Serruto et al., 2009; Sette and Rappuoli, 2010). Since *H. pylori* isolates are genetically diverse (Alm et al., 1999; Salama et al., 2000), and vaccines should preferably contain antigens that are highly conserved among different strains, the availability of complete genome sequences of independent strains may enable to select conserved antigens. There are, however, also some limitations related to the genome-based selection of vaccine candidates. For instance, this approach does not provide information about the translation and synthesis of the gene products, nor whether the deduced proteins are actually functional.

Common molecular techniques, e.g. DNA microarray technology and (immuno)proteomics, allow analyzing sets of genes and/or proteins expressed *in vivo* as well as screening of *in vivo* immunoreactive antigens. Screening sera from vaccinated mice against a *H. pylori* expression library has identified urease, the heat-shock protein (Hsp) B, putative membrane proteins and lipoprotein 20 (Lpp20) as candidate vaccine antigens (Hocking et al., 1999). Immunoproteomics is a strategy combining standard proteomics with immunological screening. Mostly, two-dimensional (2D) gel electrophoresis of *Helicobacter* proteins is performed, followed by immunoblotting with sera from *Helicobacter*-infected patients exhibiting different pathologies. Immunogenic proteins could further be characterized by mass spectrometry and sequence analysis. This technique is currently the method of choice for identifying new antigens of diagnostic and protective values (Adamczyk-Poplawska et al., 2011). It has been proposed that highly immunogenic, conserved, abundant and surface-located proteins could be used as efficient

anti-*Helicobacter* vaccine candidates (McAtee et al., 1998; Bumann et al., 2004). For instance the well-known *Helicobacter* proteins UreB, FlaA, HP-NAP and HspB have been identified as potential vaccine candidates of *H. pylori*. In almost each study, also new immunogenic proteins with potential protective value have been found. Some of them, including a putative neuraminyl-lactose binding hemagglutinin homolog (HpaA homolog) and HP0231 (disulfide bond A and C homolog) induced a protective response in the murine infection model (Sabarth et al., 2002).

Before the start of this thesis, no genomic nor (immuno)proteomic information was available for *H. suis*.

4.1.2. *In vivo* vaccination trials

Most *in vivo* vaccination experiments against gastric *Helicobacter* infections have been performed in the mouse model. It has only been possible to reliably infect mice with *H. pylori* since the mid-nineties. Therefore, in early immunization experiments, challenge was often done with *H. felis* (Nedrud, 2001). Consequently, this section is mainly focused on the effectiveness of vaccine antigens against *H. pylori* and *H. felis* in the mouse model, complemented with immunization studies against other gastric *Helicobacter* species.

As mentioned previously, initial attempts for developing a *H. pylori* vaccine were performed using inactivated or killed **whole-cell preparations** (Chen et al., 1993; Czinn et al., 1993). The antigen preparations were delivered intragastrically to mice in the presence of a mucosal adjuvant, cholera toxin (CT), and were highly effective in inducing protection against gastric *H. felis* infection (Chen et al., 1993; Czinn et al., 1993; Lee and Chen, 1994). Until today, prophylactic mucosal and parenteral immunization of mice with *Helicobacter* whole-cell lysates showed to be effective against *H. pylori* infections (Garhart et al., 2003b; Sutton et al., 2007; Flahou et al., 2009; Harbour et al., 2008; Raghavan et al., 2010). Also therapeutic immunization with whole-cell lysate may provide strong protection against both experimental *H. pylori* infection and later reinfection (Raghavan et al., 2002a; b; Nyström et al., 2006). In several of these studies a strong reduction of bacterial load was observed, even leading to complete protection in some animals.

In addition to whole-cell preparations, studies with subunit vaccines consisting of purified or recombinant proteins were carried out. Of the various candidate antigens, the most promising is

the **urease** protein, which activity is critical for the survival of *H. pylori* (Eaton et al., 1991; Tsuda et al., 1994; Karita et al., 1995). The urease subunit B (UreB) is a 65-kDa protein encoded by a 1.7-kilo base pair (kbp) gene, which frequently elicits an antibody response in patients with gastric diseases (Futagami et al., 1998; Kimmel et al., 2000). It has been suggested that the UreB seems to be more important in terms of immunogenicity and protection than the urease subunit A (UreA) (Ferrero et al., 1994; Michetti et al., 1994; Bégué et al., 2007; 2010). Table 2 provides an overview of vaccination experiments using *H. pylori* urease or its subunits, alone, or in combination with other antigens. As noted, not only the antigen, but also administration route and adjuvant or vector influence the outcome of vaccine-induced protection and immunity. Before the present PhD research, vaccination studies with *H. suis* urease had not yet been done.

Table 2. Vaccination experiments in mice against *H. pylori* or *H. felis* with urease or its subunits alone or in combination with other antigens

Antigen	Adjuvant/ vector	Delivery route	Conclusions ^a	Reference
rUrease	CT	oral, IG	• All: protection against <i>H. felis</i> challenge	Lee et al., 1995
	Freund's	SC	• Protection correlated with gastric secretory anti-urease IgA	
rUrease	CT	oral	Protection against <i>H. felis</i> challenge	Pappo et al., 1995
UreA+UreB	<i>S.typhimurium</i>	IN	• Protection against <i>H. pylori</i> challenge	Corthésy-Theulaz et al., 1998
			• Th1- and Th2 responses were generated	
UreB	<i>Lactobacillus</i>	IG	Protection against <i>H. felis</i> challenge	Corthesy et al., 2005
rUreB + rHspA	CT	oral	Combination of two proteins has a synergistic effect against <i>H. felis</i> challenge (UreB alone: 80% protection, whereas in combination 100%)	Ferrero et al., 1995
rUrease	LT	IN	• Both: protection against <i>H. pylori</i> challenge	Ermak et al., 1998
	Alum, Bay ^b	SC	• Correlation between protection and density of MHC class II T cells	
			• Antibodies not required for protection	
rUrease	LT	oral	• Both: protection against <i>H. pylori</i> challenge	Guy et al., 1998
	Bay, Saponin	SC	• Using an adjuvant inducing both Th1 and Th2 responses elicit better protection than using an adjuvant with a predominant Th2 response	
UreB	Poliovirus	oral	Prophylactic as well as therapeutic protection against <i>H. pylori</i>	Smythies et al., 2005
Urease	LT	oral, IN, rectal	Protection against <i>H. pylori</i> challenge	Kleanthous et al., 1998
Urease	LT	oral	Protection against <i>H. pylori</i> challenge	Kleanthous et al., 2001
Urease		rectal	Protection against <i>H. pylori</i> challenge + clearance	
Urease+ CagA		rectal	Protection against <i>H. pylori</i> challenge + clearance	
UreB	DNA	IN	Protection against <i>H. pylori</i> challenge	Hatzifoti et al., 2006
UreB	CpG/DNA	IN, oral, rectal, IM	No protection against <i>H. pylori</i> challenge	Zavala-Spinetti et al., 2006

UreB138 ^c	Freund's	SC	<ul style="list-style-type: none"> • Protection against <i>H. pylori</i> challenge • IgA in stomach of immunized mice were significant higher than in unimmunized mice • Severe gastritis in immunized mice 	Morihara et al., 2007
UreB	<i>rLactococcus lactis</i>	oral	<ul style="list-style-type: none"> • Protection against <i>H. pylori</i> challenge • Significant serum anti-UreB IgG and UreB-specific fecal IgA in immunized group 	Gu et al., 2009
rUreB + rHpaA _{trunc}	CT	SL/IG	<ul style="list-style-type: none"> • Gastric IL-17 correlated with protection • SL delivery induces stronger protection than IG delivery • Combination of two proteins has a synergistic effect against <i>H. pylori</i> infection 	Flach et al., 2011a
rUreB	CpG	IN	Neither immunogenic, nor protective against <i>H. pylori</i> challenge	Bégué et al., 2010
	Freund's	SC	Immunogenic and highly protective against <i>H. pylori</i> challenge	
	Alum	IM	Immunogenic and modestly protective against <i>H. pylori</i> challenge	
rUreB	CT	IN	<ul style="list-style-type: none"> • Protection against <i>H. pylori</i> challenge, which is correlated with increased specific rUreB Th17 response 	Zhang et al., 2011
UreB+CagA+VacA	attenuated <i>S.typhimurium</i>	oral	<ul style="list-style-type: none"> • Protection against <i>H. pylori</i> challenge, clearance rate of 62.5% • Protection correlated with specific Th1 response (IFN-γ), serum IgG and mucosal IgA responses 	Liu et al., 2011

^a **Conclusions:** Protection against *H. pylori/H. felis* challenge: a significant decrease of *H. pylori/H. felis* colonization in immunized and challenged groups compared to unimmunized and challenged groups. Clearance: At least some individuals are present in the immunized and challenged group with undetectable numbers of bacteria. ^b **Bay:** a glycosylamide adjuvant. ^c **UreB138:** a 138 amino acids long segment of subunit B of urease corresponding to the most important region of the enzymatic activity of *H. pylori* urease. **Abbreviations:** **r:** recombinant; **UreA** and **UreB:** urease subunit A and B, respectively; **rHspA:** recombinant heat-shock protein A; **rHpaA_{trunc}:** truncated form of recombinant *H. pylori* adhesin A; **CagA:** cytotoxin-associated protein A; **VacA:** vacuolating cytotoxin; **MHC:** major histocompatibility complex; **Alum:** aluminium hydroxide; **S.:** *Salmonella*; **CT:** cholera toxin; **LT:** heat-labile enterotoxin of *E. coli*; **SC:** subcutaneous; **SL:** sublingual; **IG:** intragastric; **IM:** intramuscular; **IN:** intranasal.

Immunization with recombinant (r)**CagA** and r**VacA** administered intragastrically together with a detoxified mutant of the heat-labile enterotoxin of *E. coli* (LT) eradicated an existing *H. pylori* infection in mice (Ghiari et al., 1997). However, a large number of *H. pylori* strains do not express CagA and VacA, thus limiting their potential as vaccine candidates if used alone. They can, however, be included in a vaccine that also contains other antigens common to all strains (see further). Another virulence factor used in immunization experiments is **HP-NAP**. Prophylactic intragastric immunization of mice with HP-NAP has been shown to induce a similar partial protection compared to vaccination with CagA and *H. pylori* lysate (Satin et al., 2000). Rossi et al. (2004) described successful therapeutic vaccination against *H. pylori* in beagle dogs with a multivalent subunit vaccine consisting of VacA, HP-NAP and CagA. Interestingly, these virulence factors have also recently been used in a Phase I study in humans (Malfertheiner et al., 2008). This study evaluated the safety and immunogenicity of a vaccine consisting of recombinant VacA, CagA, and HP-NAP given intramuscularly with an aluminium hydroxide adjuvant to *H. pylori*-negative healthy individuals. Vaccination elicited no adverse reactions and generated a humoral and T cell response and cytokine production, indicating that this is a promising candidate vaccine for further clinical studies (Malfertheiner et al., 2008).

The **HpaA** protein, which is an immunogenic adhesin of *H. pylori* (Volland et al., 2003), has also been reported to induce protection in a mouse model. Prophylactic intragastric immunization with rHpaA (+ CT) induced a reduction in bacterial colonization in some mouse strains (inbred BALB/c and outbred QS mice), but was ineffective in others (inbred C57BL/6 mice), demonstrating the importance of the host genetic background. By contrast, therapeutic immunization was effective in all three mice strains, but immunization with lysate was shown to be more effective (Sutton et al., 2007). Purification of this lipoprotein requires detergents, which might confer toxicity. Therefore, in a subsequent study, a truncated form of rHpaA (rHpaA_{trunc}), which purification does not require detergents, was created and evaluated in a mouse model (Flach et al., 2011a). The authors concluded that rHpaA_{trunc} was equally protective as rHpaA, but immunization in combination with rUreB induced significantly better protection than immunization with rHpaA_{trunc} alone. It was suggested that HpaA (and its truncated form rHpaA_{trunc}) is a promising antigen for inclusion in a mucosal vaccine against *H. pylori* (Sutton et al., 2007; Flach et al., 2011a), however most likely as a component of a multiantigenic vaccine. Recently, Anderl et al. (2009) published an abstract describing immunization against *H. pylori*

with a combination of HpaA and **GGT**. As described earlier, GGT can inhibit T cell proliferation, and thus helps to evade the host immune response. The authors found that by vaccination of mice with GGT, strong anti-GGT antibody responses block the enzymatic activity of the protein, and thereby counteract the immunosuppressive activity of GGT. As GGT is a secreted protein, GGT-specific T cells can hardly target the pathogen. Therefore, the OMP HpaA was also included in the vaccine used in this study.

Other antigens used in *H. pylori*/*H. felis* mouse vaccination studies include catalase (Radcliff, 1997), superoxide dismutase (Every et al., 2011), Lpp20 (Keenan et al., 2000), flagellar sheath proteins (Skene et al., 2007) and heat-shock proteins. The latter have been shown to induce a partial protection against *H. felis* or *H. pylori* colonization in mice (Ferrero et al., 1995; Yamaguchi et al., 2003). Heat-shock proteins could be candidates for vaccination against *H. pylori*, however like for HpaA, most probably as a component of a multiantigenic vaccine. Indeed, immunization of mice with both HspA and UreB induced a 100% protection from challenge with *H. felis*, whereas immunization with HspA alone only induced 80% reduction of bacterial load (Ferrero et al., 1995).

So far, the majority of subunit vaccines contain one or two antigens. However, some studies indicate that including more antigens might increase vaccine efficacy against *Helicobacter* infections. Wu et al. (2008) observed that a multivalent vaccine delivered either orally or intramuscularly and consisting of UreB, HspA and the surface-located protein HpaA induced a better protection against *H. pylori* in Mongolian gerbils than single-or double-antigen vaccines.

Apart from *H. felis*, only a few studies dealt with vaccination against NHPH species. Dieterich et al. (1999) showed that intranasal (+ CT) immunization of mice with recombinant *H. heilmannii* s.s. UreB or *H. pylori* urease protects mice against a *H. heilmannii* s.s. infection (prophylactic) and could also reduce the bacterial load of a preexisting infection (therapeutic). Only two studies reported immunization against *H. suis* (Hellemans et al., 2006; Flahou et al., 2009). Hellemans et al., (2006) found that intranasal (+ CT) and subcutaneous (+ saponin formulation) immunization with heterologous *H. pylori* and *H. felis* whole bacterial antigens only induced a partial protection against challenge with the at that moment unculturable “*Candidatus H. suis*”. Flahou et al. (2009) demonstrated that subcutaneous immunization of mice with whole bacterial cell lysates of *H. suis*, *H. cynogastricus* and *H. bizzozeronii* was not protective against a *H. suis* challenge.

However, prophylactic intranasal immunization of mice using a homologous (*H. suis*) or heterologous (*H. cynogastricus* and *H. bizzozeronii*) whole-cell lysate induced a protective response against *H. suis*, since several animals were negative in both a *H. suis*-specific PCR and urease activity test. This standardized intranasal mouse model can be used for future vaccination experiments against *H. suis*.

4.1.3. Mechanism of vaccine-induced *Helicobacter* immunity

Literature generally acknowledges an enhanced Th1/Th17 response to be most important in vaccine-induced protection against *H. pylori*, accompanied with innate mediators and a decreased Treg response. In addition, a role for antibody- and Th2 responses have been proposed (Blanchard and Nedrud, 2010).

The role of local and systemic antibodies in protection against gastric *Helicobacter* infections remains ambiguous. Initial and recent *H. pylori* vaccine studies showed that local IgA and IgG in the stomach were associated with protection in mice (Blanchard et al., 1995; Lee et al., 1995; Ferrero et al., 1997; Goto et al., 1999; Nyström et al., 2006; Morihara et al., 2007), and that passive immunization with IgA and IgG offered protection against *H. felis* and *H. pylori* infection (Czinn et al., 1993; Blanchard et al., 1995; Gorrell and Robins-Browne, 2009; Bhuiyan et al., 2010). In contrast to the positive evidence for antibody-mediated vaccine-induced protection against *H. pylori* infection, numerous other authors did not find a clear correlation between antibody responses and protection. Indeed, studies in B cell- or antibody- deficient mice indicated that both prophylactic and therapeutic vaccinations mediate immune protection in a B cell- and antibody-independent manner (Ermak et al., 1998; Blanchard et al., 1999; Gottwein et al., 2001; Garhart et al., 2003b; Akhiani et al., 2004).

Studies in major histocompatibility complex (MHC)-knockout mice showed that vaccine efficacy mainly depends on the presence of a MHC class II-restricted CD4⁺ T cell population (Ermak et al., 1998; Pappo et al., 1999). The role of different CD4⁺ T cell subsets in vaccination is illustrated in Figure 2. However, conflicting reports about the polarization of CD4⁺ T cell population can be found in literature. Some authors demonstrated that a Th2 or a balanced Th2/Th1 response confers protection against *H. pylori* infection (Saldinger et al., 1998; Gottwein et al., 2001), whereas most studies showed that Th2 responses are not required for protective immunity against *H. pylori* infection (Garhart et al., 2003b; Nyström and Svennerholm, 2007;

Taylor et al., 2008). Nowadays, Th1 polarized responses are considered to be more important than Th2 responses in vaccine-mediated protection (Figure 2). The role of the signature Th1 cytokine, IFN- γ , remains controversial. Several studies have reported that IFN- γ is critical for protective immunity in mice (Akhiani et al., 2002; Rahn et al., 2004; Sayi et al., 2009), whereas others found it to be less important (Sawai et al., 1999; Garhart et al., 2003a; Flach et al., 2011b). The relative importance of different cytokines in vaccine-mediated protection was compared by Flach and coworkers (2011b). Significant inverse correlations were found between bacterial colonization and gastric expression of TNF- α , IL-12p40, IFN- γ and IL-17. *In vivo* neutralization of these cytokines during the effector phase of immune response revealed a significant role of the Th17 signature cytokine IL-17, but not of TNF- α and IFN- γ in vaccine-induced protection, indicating that mainly Th17 effector mechanisms are of critical importance for protection. Indeed, recent studies demonstrated that Th17 cells are correlated with protection against *H. pylori* infection (DeLyria et al., 2009; Raghavan et al., 2010; Zhang et al., 2011). DeLyria et al. (2009) demonstrated that Th17 mediates protection in a mouse model of *H. pylori* infection, as did Velin et al. (2009) using *H. pylori* and *H. felis*. In the latter study, neutralizing IL-17 significantly reduced vaccine efficacy. As mentioned in paragraph 3.2., *H. pylori* infection may activate a Treg response in the gastric mucosa, which down-regulates both inflammation and immune response (i.e. Th1 and Th17 responses) leading to persistence of infection (Zhang et al., 2008; Kao et al., 2010). Indeed, *H. pylori*-specific-Th1 responses and protective immunity can be enhanced by depletion of Treg (Zhang et al., 2008). It has been shown that T cell activation at lymph nodes due to immunization could possibly circumvent this Treg-mediated suppression of immunity (Blanchard et al., 2004).

Finally, also innate immune mediators have been shown to play an important role in protective immunity against *H. pylori* infections. Mast cells have been shown to collaborate with CD4⁺ Th cells to mediate bacterial clearance (Velin et al., 2005), while others showed that neutrophils were essential for induction of Th17 responses (DeLyria et al., 2009). A study of Ding et al. (2009) suggested that these findings can be reconciled, as mast cells contributed to protection but did so by amplifying neutrophil recruitment and effects of IL-17. Interestingly, also DCs have been suggested to play a critical role in the development of a successful *H. pylori* vaccine (Zhang et al., 2008).

Apart from *H. felis*, nothing is known about vaccine-mediated immune response against gastric NHPH species.

Enhanced gastritis has been often observed after *H. pylori* challenge in prophylactic vaccination studies in mice. This enhanced gastritis is termed “post-immunization” gastritis and has mainly been found when using whole-cell lysate (Goto et al., 1999; Garhart et al., 2002; Morihara et al., 2007). Although this phenomenon represents a major concern in *H. pylori* vaccination trials in mice, it has been shown that the enhanced inflammation in vaccinated mice can resolve over time to background or to levels no higher than observed in infected mice (Sutton et al., 2001; Garhart et al., 2002). The mechanism of post-immunization gastritis is not fully understood. It has been shown that the severity of post-immunization gastritis is often associated with the degree of protection (Goto et al., 1999; Garhart et al., 2002; Morihara et al., 2007; Becher et al., 2010). Indeed, Becher et al. (2010) demonstrated that the vaccine-induced protection is correlated with increased properties of CD4⁺ T cells and neutrophils, and reduced numbers of Treg cells in the gastric mucosa (Becher et al., 2010).

4.2. *In vitro* antimicrobial susceptibility of gastric *Helicobacter* species

A considerable amount of work has been conducted over the last years assessing many issues around *H. pylori* eradication therapy. These studies were mainly focused on the efficacy of the current standard therapy, the investigation of antimicrobial resistance rates, and the development of rescue therapies required when treatment fails. As part of this thesis, the present overview will deal with *in vitro* antimicrobial susceptibility of *H. pylori*, complemented with that of other gastric helicobacters.

The current first choice treatment for a *H. pylori* infection consists of a proton-pump inhibitor and two antimicrobials (e.g. clarithromycin, amoxicillin, metronidazole, tetracycline or fluoroquinolones) for 7 days (Malfertheiner et al., 2007). *H. pylori* can acquire resistance to the antimicrobial agents used to treat the infection. Therefore, susceptibility testing is important in the management of the infection. The Clinical and Laboratory Standards Institute (CLSI) recognizes only the agar dilution susceptibility testing method for *H. pylori* (CLSI, 2010). This method requires a *H. pylori* cell suspension equivalent to a 2.0 McFarland standard, Mueller-Hinton plates containing 5% aged sheep blood, and incubation for 72h in a microaerobic atmosphere at 35°C +/- 2°C. Currently, the CLSI has only established a breakpoint for

clarithromycin in the case of *H. pylori* susceptibility testing (resistant breakpoint ≥ 1 $\mu\text{g/ml}$) (CLSI, 2010). Other breakpoints to define resistance have only been suggested in literature for tetracycline (≥ 2 $\mu\text{g/ml}$), ciprofloxacin (≥ 1 $\mu\text{g/ml}$), metronidazole and amoxicillin (both ≥ 8 $\mu\text{g/ml}$) (Mendonça et al., 2000; Mégraud and Lehours, 2007). *H. pylori* is intrinsically resistant to some compounds, including glycopeptides, cefsulodin, nystatin, polymyxins, nalidixic acid, trimethoprim, sulfonamides, cycloheximide and amphotericin B. Some of these are therefore used as selective agents in isolation media. A variety of other methods have been examined for their suitability for testing antimicrobial susceptibility of *H. pylori*. They include the broth dilution method (Hachem et al., 1996), the Epsilon-meter testing method (Etest) (Glupczynski et al., 2002) and the disk diffusion method (Falsafi et al., 2004). These traditional methods of susceptibility testing all require prior isolation and cultivation of *H. pylori*. Because isolation of *H. pylori* from a gastric biopsy may take several days, empiric therapy is often initiated before antimicrobial susceptibility results are available. To circumvent this problem, rapid molecular methods, including quantitative real-time PCR (qPCR) and FISH, have been developed to guide the therapy based on the presence of specific resistance determinants. Molecular rapid tests have already been developed for detection of resistance to fluoroquinolones (Glocker and Kist, 2004), tetracycline (Lawson et al., 2005) and clarithromycin (Chisholm et al., 2001; Lottspeich et al., 2007) using DNA from gastric biopsy, gastric juice or stool samples. *H. pylori* resistance is essentially due to chromosomal mutations, and for the most part, a limited number of punctual mutations are present. Genes involved in resistance to antimicrobial agents in *H. pylori* are summarized in Table 3.

Table 3. Genes involved by mutation or other genetic events leading to antimicrobial resistance in *H. pylori*

Antibiotics	Concerned gene(s)	Target	Location of action	Reference
Macrolides	<i>rrn</i> 23S	23S RNA	Point mutations at position 2142 and 2143 of 23S	Versalovic et al., 1996
Tetracyclines	<i>rrn</i> 16S	16S RNA	Change of nucleotide triplet AGA925-928→TTC	Trieber and Taylor, 2002
Quinolones	<i>gyrA</i>	DNA gyrase A	AA substitution in QRDQ mainly at 87 and 91	Moore et al., 1995
Amoxicillin	<i>pbp-1a</i>	Penicillin-binding protein	AA substitution Ser-414→Arg	Gerrits et al., 2002
Metronidazole	<i>rdxA</i> , <i>frxA</i>	Oxygen-insensitive nitroreductase, flavin oxidoreductase	Different mutations in <i>rdxA</i> and/or <i>frxA</i>	Goodwin et al., 1998; Jeong et al., 2000
Rifampicins	<i>rpoB</i>	DNA-dependent RNA polymerase subunit B	Mutations at positions 524, 525, 585 of <i>rpoB</i>	Heep et al., 1999

AA: amino acid, QRDQ: quinolone-resistance determining region.

There are no standard treatment schemes for eradication of gastric NHPH. Therefore, infections with NHPH in humans have been empirically treated with similar therapeutic regimens as used for *H. pylori* (Solnick, 2003). Triple therapy using combinations of a proton pump inhibitor and two antimicrobial agents (clarithromycin, amoxicillin, metronidazole or tetracycline) for 7 to 14 days have been shown to be effective (Goddard et al., 1997; Ojano et al., 2012; Joosten et al., 2013). However, also these species may acquire antimicrobial resistance. Indeed, an eradication therapy regimen containing lansoprazole (proton pump inhibitor), tetracycline, and metronidazole was not successful in a patient infected with *H. bizzozeronii*, as shown by histology and positive culture. *In vitro* susceptibility testing before and after treatment revealed high minimal inhibitory concentrations (MICs) for metronidazole (Kivistö et al., 2010).

To date, *in vitro* susceptibility studies have only been performed for *H. bizzozeronii*, *H. salomonis* and *H. felis*, three natural inhabitants of the gastric mucosa of cats and dogs (Van den Buck et al., 2005b). MICs were determined using a slightly modified method (that is horse blood instead of sheep blood) from that recommended for *H. pylori* and *Campylobacter jejuni*. All strains showed to be highly susceptible to ampicillin, clarithromycin, tetracycline, tylosin, enrofloxacin, gentamicin and neomycin. Acquired resistance was only detected to metronidazole

in one *H. bizzozeronii* and two *H. felis* strains, showing a MIC of 16 µg/ml (Van den Buck et al., 2005b). Only since a few years, a successful *in vitro* cultivation method has been developed for *H. suis* (Baele et al., 2008). Hence, before the present PhD thesis, *in vitro* antimicrobial susceptibility studies for this species had not been performed.

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SCIENTIFIC AIMS

Worldwide, *Helicobacter (H.) suis* causes chronic gastritis and decreased daily weight gain in pigs. This microorganism has also been associated with ulcers of the pars oesophagea of the porcine stomach. In addition, it is the most frequent non-*H. pylori* *Helicobacter* species in humans suffering from gastric disorders. For a long time, the lack of pure *H. suis* *in vitro* isolates hampered the progress of research on this bacterium. Only in 2008, a successful *in vitro* culture method was developed, creating new opportunities to investigate the pathogenesis of *H. suis* infections and to develop control strategies.

In contrast to *H. pylori*, little is known about the pathogenesis of *H. suis* infections, especially from a molecular perspective. Therefore, the genome of *H. suis* was sequenced with the aim to identify genes possibly involved in gastric colonization, persistence and pathogenicity of this microorganism (**first aim** of this thesis, Chapter 1).

Measures to control *H. suis* infections in pigs may not only decrease the number of pigs suffering from gastric disease, but are also important from a public health point of view. When we started our studies, the few vaccination trials that had been carried out for *H. suis* only focused on the protective efficacy of whole-cell preparations. However, it was not known which bacterial proteins are important for induction of protective immunity against *H. suis* infections. Therefore, **the second aim** of this thesis was to obtain better insights into this matter. First, it was determined which *H. suis* proteins are recognized by sera from protected animals and not by sera from non-protected animals. Subsequently, the protective efficacy of a limited number of candidate antigens was evaluated and compared with that of *H. suis* whole-cell lysate in a standardized mouse model (Chapters 2 and 3).

Currently, *H. suis* infections in human patients suffering from severe gastric pathology are treated in the same way as *H. pylori* infections. However, little is known about intrinsic and acquired antimicrobial resistance of *H. suis* isolates. Therefore, **the third aim** of this PhD thesis was to develop an *in vitro* assay allowing the determination of the antimicrobial susceptibility of *H. suis* isolates (Chapter 4).

EXPERIMENTAL STUDIES

CHAPTER 1

Genome sequence of *Helicobacter suis* supports its role in gastric pathology

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Abstract

Helicobacter (H.) suis has been associated with chronic gastritis and ulcers of the pars oesophagea in pigs, and with gastritis, peptic ulcer disease and gastric mucosa-associated lymphoid tissue lymphoma in humans. In order to obtain better insight into the genes involved in pathogenicity and in the specific adaptation to the gastric environment of *H. suis*, a genome analysis was performed of two *H. suis* strains isolated from the gastric mucosa of swine. Homologs of the vast majority of genes shown to be important for gastric colonization of the human pathogen *H. pylori* were detected in the *H. suis* genome. *H. suis* encodes several putative outer membrane proteins, of which two similar to the *H. pylori* adhesins HpaA and HorB. *H. suis* harbours an almost complete *comB* type IV secretion system and members of the type IV secretion system 3, but lacks most of the genes present in the *cag* pathogenicity island of *H. pylori*. Homologs of genes encoding the *H. pylori* neutrophil-activating protein and γ -glutamyl transpeptidase were identified in *H. suis*. *H. suis* also possesses several other presumptive virulence-associated genes, including homologs for *mviN*, the *H. pylori* flavodoxin gene, and a homolog of the *H. pylori* vacuolating cytotoxin A gene. It was concluded that although genes coding for some important virulence factors in *H. pylori*, such as the cytotoxin-associated protein (CagA), are not detected in the *H. suis* genome, homologs of other genes associated with colonization and virulence of *H. pylori* and other bacteria are present.

Introduction

Helicobacter (H.) suis is a very fastidious, spiral-shaped, Gram-negative bacterium requiring a biphasic culture medium at pH 5 enriched with fetal calf serum, and a microaerobic atmosphere for *in vitro* growth (Baele et al., 2008). *H. suis* colonizes the stomach of more than 60% of slaughter pigs (Baele et al., 2008; Hellemans et al., 2007b). Although the exact role of *H. suis* in gastric disease in pigs is still unclear, it has been associated with chronic gastritis (Mendes et al., 1991; Hellemans et al., 2007a) and ulcers of the pars oesophagea of the stomach (Queiroz et al., 1996; Roosendaal et al., 2000; Haesebrouck et al., 2009). This may result in significant economic losses due to sudden death, decreased feed intake and reduced daily weight gain (Ayles et al., 1996). A reduction of approximately 20 g/day in weight gain was observed in animals experimentally infected with *H. suis*, compared to the non-infected control animals (Kumar et al., 2010a).

Bacterial gastric disorders in humans are mainly caused by *Helicobacter pylori* (Cover and Blaser, 2009). However, non-*Helicobacter pylori* helicobacters (NHPH) have also been associated with human gastric disease with a prevalence ranging between 0.2 and 6% (Haesebrouck et al., 2009). *H. suis* is the most frequent NHPH species found in humans, where it was originally named “*H. heilmannii*” type 1 (O’Rourke et al., 2004). There are strong indications that pigs may serve as a source of infection for humans (Meining et al., 1998; Haesebrouck et al., 2009). In the human host, *H. suis* has been associated with peptic ulcer disease (Debognie et al., 1998), gastric mucosa-associated lymphoid tissue (MALT) lymphoma (Morgner et al., 1995) and chronic gastritis (Debognie et al., 1995). In rodent models of human gastric disease, the bacterium causes severe inflammation and MALT lymphoma-like lesions (Flahou et al., 2010).

Up to now, little is known about the pathogenesis of *H. suis* infections. To improve understanding in the genes playing a role in pathogenicity, gastric colonization and persistence of *H. suis*, a genome-wide comparison with the well-investigated *H. pylori* genome was performed. Some virulence factors may indeed be similar for both bacteria. As there may also be differences, *ab initio* annotations of the *H. suis* genome were performed as well.

Materials and methods

Genome sequencing

A pyrosequencing (454 Life Sciences Corporation, Branford, CT, USA) assay was applied to the genome of the type strain of *H. suis* (HS1^T = LMG 23995^T = DSM 19735^T) and *H. suis* strain 5 (HS5), isolated from the gastric mucosa of two different swine, according to the method described by Baele et al. (2008). Quality filtered sequences were assembled into contigs using a 454 Newbler assembler (Roche, Branford, CT, USA).

Functional annotation

In order to maximize the number of quality gene annotations, two different annotating approaches were followed: cross-mapping with three *Helicobacter pylori* strains (26695, Shi470, and G27 with NCBI accession numbers NC_000915, NC_010698, and NC_011333, respectively), and *ab initio* annotation.

Cross-mapping annotation

A custom BLAST (Altschul et al., 1997) database was created from the HS1^T and HS5 genomic contigs. The *H. pylori* proteome and non-coding RNAs were aligned (tblastn program of the BLAST suite, e threshold set to 10^{-3}) to the *H. suis* database. For each BLAST hit the following additional information was analysed: 1) (secretion) signal peptide cleavage site if present, as assessed by the SignalP 3.0 program (Nielsen et al., 1997; Bendtsen et al., 2004); 2) specifications of transmembrane helices (number, start and end positions, presumed topology with regard to the cytoplasmic membrane) if present, as assessed by the TMHMM program (Krogh et al., 2001); 3) an estimate of the ribosome binding strength of the mRNA region preceding the most probable start codon. Ribosome binding strength was estimated by applying two established facts: i) on an mRNA strand, usually within 20 nucleotides before the actual start codon, the reverse complement of 5 to 7 nucleotides near the 16S rRNA 3' end acts as an attractor and positioner for the ribosomal small subunit; this region is known as the Shine-Dalgarno sequence (Shine and Dalgarno, 1974; Mikkowen et al., 1994); ii) in Gram-negative bacteria an AU-rich mRNA region some 16 nucleotides long and immediately preceding the Shine-Dalgarno sequence may also attract and position ribosomes to help initiate translation of the correct, biologically active gene product (Boni et al., 1991; Komarova et al., 2005). For *H. suis*, the Shine-Dalgarno sequence was determined to be a subsequence of AGGAGGU (which is

the reverse complement of the 3' end of the 16S rRNA), and the minimum AU-richness (equivalent to ribosome binding capacity) of the preceding region was arbitrarily set to 10/16. For each theoretical ORF a range of possible start codons was scored; the higher the similarity to the ideal Shine-Dalgarno sequence, or the AU-richer the preceding region, or the better a combination of both, the more likely the potential start codon is to be the actual start codon.

***Ab initio* annotation**

For *ab initio* annotation, theoretical open reading frames (ORFs) were first determined using the EMBOSS getorf tool (with minimum ORF length set to 90 nucleotides, and taking all alternative start codons into account) (Rice et al., 2000). All ORFs were translated subsequently, and BLAST (blastp program) was performed with an e threshold of 10^{-15} against the Uniprot-KB universal protein database. The generalist algorithm of getorf yielded roughly a tenfold of the expected natural ORFs, reducing the risk of false negatives. In order to keep the false positive rate low, extra parameters were considered: 1) percentage alignment between query and hit ORFs; 2) percentage similarity or conservation between aligned portions of query and hit ORFs; 3) ribosome binding strength (for more details see above). To determine the presence of one or more conserved domains a rpsblast search (with default parameter values) was carried out for every single theoretical ORF against the compiled Conserved Domain Database which holds protein domain alignments from several other database sources (Marchler-Bauer et al., 2009).

Results

General features of the *H. suis* genome

In the HS1^T genome a total of 1 635 292 base pairs and in the HS5 genome 1 669 960 bp were sequenced, both with an average GC content of 40%. In contrast to *H. pylori*, only one copy of both the 16S and 23S rRNA genes was detected, but like *H. pylori*, *H. suis* has three copies of the 5S rRNA gene. Thirty-eight transfer RNAs were identified. On the whole, 1266 ORFs from HS1^T and 1257 from HS5 were detected, of which 194 and 191 encoded hypothetical proteins respectively. In 98 and 92 ORFs a signal peptide cleavage site was detected, demonstrating predicted secreted proteins of HS1^T and HS5 respectively. The TMHMM program predicted 210 and 206 proteins with at least one transmembrane helix for HS1^T and HS5 respectively. The

sequence fraction identical for HS1^T and HS5 is henceforward described together as the “*H. suis* genome”.

Genes possibly involved in gastric colonization and persistence

Homologs of *H. pylori* genes involved in acid acclimation, chemotaxis, adhesion to gastric epithelial cells, oxidative stress resistance (Table 1), and motility were detected in the *H. suis* genome. The latter were identified as a flagellar biosystem similar to that of *H. pylori* (Tomb et al., 1997). Moreover, *H. suis* contains a fibrinogen/fibrin-binding protein coding gene, but the corresponding protein lacks a transmembrane helix or signal peptide cleavage site according to the bioinformatics tools mentioned earlier. Homologs coding for CMP-N-acetylneuraminic acid synthetase (NeuA) (HSUHS1_0474, HSUHS5_0481), sialic acid synthase (NeuB) (HSUHS1_0477, HSUHS5_0478), and UDP-N-acetylglucosamine-2-epimerase (WecB) (HSUHS1_1107, HSUHS5_0784) were observed as well.

Genes encoding putative outer membrane proteins (OMPs) in relation to *H. pylori* OMPs are presented in Additional file 1. Genes coding for members of major *H. pylori* OMP families (Hop, Hor, Hof proteins, iron-regulated and efflux pump OMPs) could be aligned with the *H. pylori* genome. Both *H. suis* strains contain the *hof* genes *hofA*, *C*, *E*, *F*, the *hop* genes *hopE*, *G-2* and *H*, and the *hor* genes *horB*, *C*, *D*, and *J*. Additionally, HS1^T contains homologs of the *hopW* protein precursor and *horE*, whereas HS5 possesses additional homologs of *horA*, *horF*, and *horL*. No members of the *Helicobacter* outer membrane (*hom*) family were detected in *H. suis*. Besides the major *H. pylori* OMP family proteins, the *H. suis* genome contains some predicted OMPs based on their N-terminal pattern of alternating hydrophobic amino acids similar to porins, encompassing *omp29* for HS1^T and *omp11* and *omp29* for HS5. A 491 amino acids membrane-associated homolog of the virulence factor MviN, aligned for 92% with the MviN homolog of *H. acinonychis* (Hac_1250), was also present in *H. suis*.

Type IV secretion systems in *H. suis*

Of the *H. pylori* type IV secretion systems (T4SS), only two members of the *cag* pathogenicity island (*cagPAI*) were identified in the *H. suis* genome (*cag23/E* and *cagX*). Most members of the *comB* transport apparatus were present. These include *comB2*, *B3*, *B6*, *B8* and a number of additional genes not classified as *comB*: *recA*, *comE*, *comL* and *dprA*. *H. suis* possesses genes encoding VirB- and VirD-type ATPases (*virB4*, *B8*, *B9*, *B10*, *B11*, and *virD2*, *D4*), all designated

members of the *H. pylori* type IV secretion system 3 (*tfs3*). The HS1^T and HS5 T4SS are presented in Table 2.

Genes possibly involved in induction of gastric lesions

Homologs of *H. pylori* genes involved in induction of gastric lesions in the *H. suis* genome are summarized in Table 3. Homology searches with the *H. pylori* vacuolating cytotoxin A gene (*vacA*) identified HSUHS1_0989 in HS1^T. The corresponding protein, which is exceptional in that it is one of the longest in the world of prokaryotes, possesses three small conserved VacA regions (residues 490-545, 941-995, and 1043-1351), followed by an autotransporter region (residues 2730-2983). The amino acid sequence of the HS5 homolog (HSUHS5_0761) could be aligned for 22% with the *H. pylori* strain HPAG1 sequence, and possesses only one conserved VacA region (residues 242-298), followed by an autotransporter region (1258-1510). In both *vacA* homologs, no signal sequence was determined. Additionally, an ulcer-associated adenine-specific DNA methyltransferase (HSUHS1_0375, HSUHS5_0957) coding sequence was identified, whereas a molecular homolog of the ulcer-associated restriction endonuclease (*iceA*) could not be discovered in *H. suis*. *H. suis* contains homologs of *pgbA* and *pgbB* encoding plasminogen-binding proteins, though both lacking a transmembrane helix or signal peptide cleavage site according to the bioinformatics tools mentioned earlier. *H. suis* harbours homologs of genes coding for the *H. pylori* neutrophil-activating protein (HP-NAP) and γ -glutamyl transpeptidase (HP-GGT). Homologs encoding the *H. pylori* flavodoxin *fldA* and the pyruvate-oxidoreductase complex (POR) members *porA*, *porB*, *porC*, and *porD* were also identified in *H. suis*.

Discussion

Genes possibly involved in gastric colonization and persistence

The results of the present study demonstrate that several *H. pylori* genes involved in acid acclimation, chemotaxis and motility, have counterparts in the *H. suis* genome. These genes are known to be essential for colonization of the human gastric mucosa (Eaton et al., 1991; Tomb et al., 1997; Skouloubris et al., 1997; 2001; Wen et al., 2003).

Several OMP coding sequences were identified by comparative analyses with *H. pylori* and other bacterial species. *H. suis* contains some similar members of the major OMP families described in *H. pylori* (Alm et al., 2000). Some of these OMPs have been described to be involved in adhesion of *H. pylori* to the gastric mucosa, which is widely assumed to play an important role in the initial colonization and long-term persistence in the human stomach. These include the gastric epithelial cell adhesin HorB (Snelling et al., 2007) and the surface lipoprotein, *H. pylori* adhesin A (HpaA). HpaA, also annotated as neuraminyllactose-binding hemagglutinin, is found exclusively in *Helicobacter* and binds to sialic acid-rich macromolecules present on the gastric epithelium (Carlsohn et al., 2006). On the other hand, *H. suis* lacks homologs of several other *H. pylori* adhesion factors, including genes coding for the blood group antigen-binding adhesins *babA* (*hopS*) and *babB* (*hopT*), the sialic acid-binding adhesins *sabA* (*hopP*) and *sabB* (*hopO*), and the adherence-associated lipoproteins *alpA* (*hopC*) and *alpB* (*hopB*) (Odenbreit et al., 2009).

H. suis contains a fibrinectin/fibrinogen-binding protein coding gene, which may enhance its adherence to injured gastric tissue. Damage to host epithelial cells may indeed expose fibronectin and other extracellular matrix components. Strong homology was found with fibronectin-binding proteins of *H. felis* (YP_004072974), *H. canadensis* (ZP_048703091) and *Wolinella succinogenes* (NP_907753). To our knowledge, no exact function has been given to these proteins in these species. In *Campylobacter jejuni*, however, fibronectin-binding proteins CadF and FlpA have been shown to be involved in adherence to and/or invasion of host's intestinal epithelial cells (Monteville et al., 2003; Konkel et al., 2010). According to the bioinformatics tools used here, the *H. suis* fibronectin-binding protein lacks a transmembrane helix or signal peptidase cleavage site, indicating that it is not surface exposed or secreted. Its real role in colonization therefore remains to be elucidated.

Three genes involved in sialic acid biosynthesis (*neuA*, *neuB*, and *wecB*) were annotated in the *H. suis* genome, indicating that this bacterium may decorate its surface with sialic acid. The presence of surface sialylation has been studied extensively in pathogenic bacteria, where it contributes to evasion of the host complement defense system (Severi et al., 2007).

Additionally, *H. suis* possesses genes encoding enzymes involved in oxidative-stress resistance (*napA*, *sodB*, *kata*, *mutS*, *mdaB*, and peroxiredoxin coding sequence). This indicates that *H. suis* may harbour a defense mechanism against the host inflammatory response, contributing to the ability of chronic gastric colonization by this bacterium (Wang et al., 2006).

Type IV secretion systems in *H. suis*

Two partial T4SS were predicted in the *H. suis* genome, namely the *comB* cluster and the *tfs3* system. The *H. suis comB* system probably plays a role in genetic transformation (Hofreuter et al., 2001; Karnholz et al., 2006). Transformation of DNA can be responsible for the high degree of diversity among *H. suis* strains as has been recently demonstrated by multilocus sequence typing of available *H. suis* strains (Kumar et al., 2010b). The role of the *H. pylori tfs3* secretion system in pathogenesis is not exactly known. Seven genes of the *tfs3* cluster are homologs of genes involved in type IV secretion: *virB4*, *virB11*, and *virD4* code for ATPases which move substrates to and through the pore. The latter is coded by transmembrane pore genes *virB7*, *virB8*, *virB9*, and *virB10* (Kersulyte et al., 2003). All these genes, except *virB7* were identified in *H. suis*, indicating that the *H. suis tfs3* can be important in transmembrane transport of substrates in *H. suis*.

The *H. pylori cag* pathogenicity island (*cagPAI*) region encodes a T4SS allowing *H. pylori* to insert the cytotoxin-associated antigen A (CagA) into the host cell. This process results in altered host cell structure, an increased inflammatory response, and a higher risk for gastric adenocarcinoma (Backert and Selbach, 2008). Although *H. suis* possesses two members of the *H. pylori cagPAI* (*cag23/E* and *cagX*), the majority of genes, including the gene coding for pathology-causing protein (CagA), were not identified. This indicates that HS1^T and HS5 lack a functional *cag* protein transporter secretion system.

Genes possibly involved in induction of gastric lesions

Genomic comparison of *H. suis* with *H. pylori* resulted in the identification of additional genes possibly associated with virulence in *H. suis*. A *H. suis* homolog of the *H. pylori vacA* was detected. VacA is both a cytotoxin of the gastric epithelial cell layer, and an immunomodulatory toxin of *H. pylori* (Gebert et al., 2004). *H. pylori* contains either a functional or non-functional *vacA*. The *H. suis vacA* homolog exhibits no *vacA* signal sequence, indicating that it might encode a non-functional cytotoxin (Atherton et al., 1995). *In vitro* and *in vivo* studies with a knockout mutant of the *H. suis vacA* could clarify the functionality of the *vacA* homolog in this *Helicobacter* species.

Strong homology was found with two *H. pylori* virulence-associated genes namely *napA*, encoding the HP-NAP and *ggt*, encoding HP-GGT. The *H. pylori* GGT has been identified as an

apoptosis-inducing protein (Shibayama et al., 2003; Kim et al., 2007). The HP-NAP protein is designated as a proinflammatory and immunodominant protein by stimulating production of oxygen radicals and IL-12 from neutrophils and recruiting leukocytes in vivo (Brisslert et al., 2005; Wang et al., 2008). Moreover, HP-NAP also plays a role in protecting *H. pylori* from oxidative stress by binding free iron (Cooksley et al., 2003). *H. suis* contains homologs of two *H. pylori* genes coding for plasminogen-binding proteins, *pgbA* and *pgbB*. The corresponding proteins, PgbA and PgbB bind host plasminogen, which subsequently can be activated to plasmin and may contribute to obstructing the natural healing process of gastric ulcers (Ljung, 2000; Jönsson et al., 2004). The biological role of the *H. suis* *pgbA* and *B* homologs in chronicity of gastric ulceration is uncertain, as no exact membrane association was found in the corresponding proteins.

The risk to develop MALT lymphomas in *H. suis* infected human patients is higher than after infection with *H. pylori* (Morgner et al., 1995; Haesebrouck et al., 2009). Homologs encoding the *H. pylori* flavodoxin (*fldA*) and its electron donor, the POR enzyme complex (*porA* to *D*) were found in *H. suis*. The *H. pylori* flavodoxin protein (FldA) has been proposed to play a role in the pathogenesis of *H. pylori*-associated MALT lymphoma, as antibodies against the *H. pylori* FldA protein were more prevalent in patients with MALT lymphomas compared to patients with other *H. pylori*-related diseases (Chang et al., 1999). Besides, insertion mutagenesis of the *fldA* and the *por* complex has shown that these genes are essential for the survival of *H. pylori* (Freigang et al., 2001). These observations indicate that *fldA* and its *por* complex may play a role in gastric colonization of *H. suis* and MALT lymphoma development in *H. suis* infected people.

Recently, the genomes of the carcinogenic *H. pylori* strain B38 and the carcinogenic and ulcerogenic *Helicobacter mustelae* have been sequenced (O'Toole et al., 2010; Thiberge et al., 2010). Both helicobacters lack homologs of major *H. pylori* virulence genes (e.g. *cagA*, *babA/B*, *sabA/B*), which are also absent in the *H. suis* genome. Additionally, *H. mustelae* lacks a *vacA* homolog. Despite this absence, infection with *H. pylori* strain B38 and *H. mustelae* has been associated with gastric MALT lymphomas and other gastric disorders. Whole genome sequencing data are also available from *H. acinonychis* strain Sheeba, a gastric pathogen of large felines. Similar to *H. suis*, *H. acinonychis* lacks a *cagPAI* as well as genes encoding BabA/B and SabA/B. Both species contain a *vacA* homolog, which for *H. acinonychis* has been described to be fragmented (Dailidiene et al., 2004; Eppinger et al., 2006).

H. suis contains a *mviN* homolog. This gene has been described to be a virulence factor of several bacterial species, such as *Burkholderia pseudomallei* and *Vibrio alginolyticus* (Ling et al., 2006; Cao et al., 2010). In addition to virulence, MviN has been described to be essential for *in vitro* growth of these and other bacteria (Ling et al., 2006; Inoue et al., 2008; Cao et al., 2010). The biological significance of *mviN* in the *Helicobacter* genus, however, remains to be elucidated.

Conclusion

Although *H. suis* lacks homologs of some major *H. pylori* virulence genes, other candidate virulence factors, such as *napA*, *ggt*, *mviN*, and *fldA* were detected. *H. suis* also possesses genes known to be essential for gastric colonization. Future *in vitro* and *in vivo* research of the currently presented genes of this porcine and human gastric pathogen should elucidate their precise role in colonization and virulence.

Nucleotide sequence accession numbers

The genome sequences have been deposited at GenBank/EMBL/DDBJ under the accession ADGY000000000 for HS1^T and ADHO000000000 for HS5. The versions described in this paper are the first versions, ADGY1000000 and ADHO1000000.

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Table 1. Genes associated with pH homeostasis, chemotaxis, adhesion to epithelial cells, and oxidative stress resistance in the genome of *H. suis* type strain 1 (HS1^T) and *H. suis* strain 5 (HS5)

Group	Gene detected in HS1 ^T	Gene detected in HS5	Description of homolog	Percentage of sequence aligned (of which % conserved) with described homolog ¹
pH homeostasis	HSUHS1_0708	HSUHS5_0286	Urease subunit alfa (<i>ureA</i>) of <i>H. heilmannii</i>	100 (94)
	HSUHS1_0707	HSUHS5_0285	Urease subunit beta (<i>ureB</i>) of <i>H. heilmannii</i>	100 (94)
	HSUHS1_0706	HSUHS5_0284	Urease transporter (<i>ureI</i>) of <i>H. felis</i>	100 (89)
	HSUHS1_0705	HSUHS5_0283	Urease accessory protein (<i>ureE</i>) of <i>H. bizzozeronii</i>	100 (84)
	HSUHS1_0704	HSUHS5_0282	Urease accessory protein (<i>ureF</i>) of <i>H. bizzozeronii</i>	100 (84)
	HSUHS1_0702	HSUHS5_0280	Urease accessory protein (<i>ureH</i>) of <i>H. bizzozeronii</i>	96 (84)
	HSUHS1_0703	HSUHS5_0281	Urease accessory protein (<i>ureG</i>) of <i>H. bizzozeronii</i>	100 (95)
	HSUHS1_0133	HSUHS5_0547	Hydrogenase expression/formation protein (<i>hypA</i>) of <i>H. pylori</i>	98 (83)
	HSUHS1_0615	HSUHS5_0817	Hydrogenase expression/formation protein (<i>hypB</i>) of <i>H. pylori</i>	99 (91)
	HSUHS1_0616	HSUHS5_0816	Hydrogenase expression/formation protein (<i>hypC</i>) of <i>H. pylori</i>	98 (89)
	HSUHS1_0617	HSUHS5_0815	Hydrogenase expression/formation protein (<i>hypD</i>) of <i>H. achinonychis</i>	98 (80)
	HSUHS1_0081	HSUHS5_1197	l-Asparaginase II (<i>ansB</i>) of <i>H. pylori</i>	98 (64)
	HSUHS1_0230	HSUHS5_1130	Arginase (<i>rocF</i>) of <i>H. pylori</i>	99 (75)
	HSUHS1_0888	HSUHS5_0231	Acylamide amidohydrolase (<i>amiE</i>) of <i>H. pylori</i>	100 (93)
	HSUHS1_0680	HSUHS5_0265	Formamidase (<i>amiF</i>) of <i>H. pylori</i>	100 (98)
	HSUHS1_0161	HSUHS5_1077	α -Carbonic anhydrase of <i>H. pylori</i>	92 (69)
	HSUHS1_0391	HSUHS5_0874	Aspartase (<i>aspA</i>) of <i>H. acinonychis</i>	100 (89)

Chemotaxis	HSUHS1_1004	HSUHS5_0649	CheA-MCP interaction modulator of <i>H. pylori</i>	99 (79)
	HSUHS1_1003	-	Bifunctional chemotaxis protein (<i>cheF</i>) of <i>H. pylori</i>	82 (86)
	HSUHS1_1002	HSUHS5_0775	Purine-binding chemotaxis protein (<i>cheW</i>) of <i>H. pylori</i>	98 (91)
	HSUHS1_0538	HSUHS5_0706	Chemotaxis protein (<i>cheV</i>) of <i>H. pylori</i>	100 (92)
	HSUHS1_0846	HSUHS5_0081	Putative chemotaxis protein of <i>H. pylori</i>	100 (79)
	HSUHS1_0299	HSUHS5_0250	Chemotaxis protein (<i>cheY</i>) of <i>H. pylori</i>	100 (95)
	HSUHS1_1001	HSUHS5_0774	Methyl-accepting chemotaxis protein (<i>tlpA</i>) of <i>H. pylori</i>	100 (60)
	HSUHS1_0286	HSUHS5_0256	Methyl-accepting chemotaxis protein (<i>tlpB</i>) of <i>H. pylori</i>	98 (63)
	HSUHS1_0479	HSUHS5_0476	Methyl- accepting chemotaxis protein of <i>H. acinonychis</i>	100 (66)
	HSUHS1_0196	HSUHS5_0122	Methyl- accepting chemotaxis protein of <i>Campylobacter upsaliensis</i> ²	99 (53)
	HSUHS1_0141	HSUHS5_0641	Methyl- accepting chemotaxis protein of <i>Campylobacter fetus</i> subsp. <i>fetus</i> ²	99 (64)
	HSUHS1_0763	-	Methyl- accepting chemotaxis protein of <i>Methylibium petroleiphilum</i> ²	83 (52)
	HSUHS1_0944	HSUHS5_0990	Methyl-accepting chemotaxis sensory transducer <i>Marinomonas</i> sp. ²	57 (59)
Adhesion	HSUHS1_0666	HSUHS5_1053	Outer membrane protein (<i>horB</i>) of <i>H. pylori</i>	100 (63)
	HSUHS1_0354	HSUHS5_0398	Neuraminylactose-binding hemagglutinin (<i>hpaA</i>) of <i>H. acinonychis</i>	94 (77)
Oxidative stress resistance	HSUHS1_1147	HSUHS5_0608	Catalase (<i>katA</i>) of <i>H. acinonychis</i>	95 (82)
	HSUHS1_0549	HSUHS5_1206	Mismatch repair ATPase (<i>mutS</i>) of <i>H. hepaticus</i>	99 (60)
	HSUHS1_0163	HSUHS5_0495	Superoxide dismutase (<i>sodB</i>) of <i>H. pylori</i>	100 (90)
	HSUHS1_1186	HSUHS5_0005	Bacterioferritin co-migratory protein of <i>H. hepaticus</i>	99 (72)
	HSUHS1_0683	HSUHS5_0262	NAD(P)H quinone reductase (<i>mdaB</i>) of <i>Campylobacter fetus</i> subsp. <i>fetus</i>	97 (68)
	HSUHS1_0689	HSUHS5_0268	Peroxiredoxin of <i>H. pylori</i> ³	100 (92)

¹ Resulting from tblastn-based cross-mapping of the *H. pylori* proteome to the *H. suis* HS1^T and HS5 genomes and blastp-based *ab initio* analyses of the translated *H. suis* HS1^T and HS5 ORFs against the Uniprot-KB universal protein database. Differences between HS1^T and HS5 homologs $\leq 1\%$. ² Lacking in other *Helicobacter* genomes available at GenBank. ³ Member of the 2-Cys peroxiredoxin superfamily.

Table 2. *H. suis* strain 1 (HS1^T) and strain 5 (HS5) homologs of *H. pylori* and other *Helicobacter* sp. type IV secretion system genes

Homolog	Gene detected in HS1 ^T	Gene detected in HS5	Description of corresponding protein	Percentage of sequence fraction aligned (of which % conserved) with <i>Helicobacter</i> homolog ¹
<i>cag</i> pathogenicity island				
<i>cag23/E</i> of <i>H. pylori</i>	HSUHS1_0731	HSUHS5_1234	DNA transfer protein	81 (42)
<i>cagX</i> of <i>H. pylori</i>	HSUHS1_0964	HSUHS5_0688	Conjugal plasmid transfer protein	92 (71)
<i>comB</i> system				
<i>comB2</i> of <i>H. acinonychis</i>	HSUHS1_1181	HSUHS5_0010	ComB2 protein	96 (64)
<i>comB3</i> of <i>H. acinonychis</i>	HSUHS1_1182	HSUHS5_0009	ComB3 competence protein	95 (77)
<i>comB6</i> of <i>H. pylori</i>	HSUHS1_0337	-	NADH-ubiquinone	70 (85)
<i>comB8</i> of <i>H. pylori</i>	HSUHS1_0747	Overlap with <i>virB8</i>	oxidoreductase	93 (66)
<i>trbL</i> of <i>H. pylori</i>	HSUHS1_0755	HSUHS5_0054	comB8 competence protein	99 (77)
<i>comE</i> of <i>H. acinonychis</i>	HSUHS1_0314	HSUHS5_0381	TrbL protein	94 (55)
<i>comL</i> of <i>H. pylori</i>	HSUHS1_0722	HSUHS5_0300	Competence locus E	99 (84)
<i>dprA</i> of <i>H. acinonychis</i>	HSUHS1_0096	HSUHS5_0824	Competence protein	99 (70)
<i>recA</i> of <i>H. hepaticus</i>	HSUHS1_0672	HSUHS5_1058	DNA processing protein Recombinase A	97 (84)
<i>virB</i> –homologs				
<i>virB4</i> of <i>H. pylori</i>	HSUHS1_0960	HSUHS5_0692	DNA transfer protein	98 (68)
<i>virB8</i> of <i>H. pylori</i>	HSUHS1_0963	HSUHS5_0689	DNA transfer protein	91 (61)
<i>virB9</i> of <i>H. cetorum</i>	HSUHS1_0319	-	VirB9 protein	76 (69)
<i>virB10</i> of <i>H. cetorum</i>	HSUHS1_0320	-	VirB10 protein	90 (77)
putative <i>virB9</i> of <i>H. pylori</i>	-	HSUHS5_0372	Putative VirB9 protein	100 (86)
putative <i>virB10</i> of <i>H. pylori</i>	-	HSUHS5_0371	Putative VirB10 protein	97 (87)
<i>virB11</i> of <i>H. pylori</i>	HSUHS1_0750	HSUHS5_0368	VirB11 protein	100 (98)
<i>virB11</i> of <i>H. cetorum</i>	HSUHS1_0965	-	VirB11 protein	95 (71)
<i>virB11</i> -like of <i>H.pylori</i> (HPSH_04565)	-	HSUHS5_0686	VirB11-like protein	98 (72)
<i>virB11</i> -like of <i>H.pylori</i> (HPSH_07250)	HSUHS1_0036	HSUHS5_0600	Type IV ATPase	100 (75)
<i>virD</i> – homologs				
<i>virD2</i> of <i>H. cetorum</i>	HSUHS1_0752	HSUHS5_0414	VirD2 protein (relaxase)	100 (90)
<i>virD4</i> of <i>H.pylori</i>	HSUHS1_0870	HSUHS5_0257	VirD4 protein (conjugation protein)	82 (78)

¹ Resulting from tblastn-based cross-mapping of the *H. pylori* proteome to the *H. suis* HS1^T and HS5 genomes and blastp-based *ab initio* analyses of the translated *H. suis* HS1^T and HS5 ORFs against the Uniprot-KB universal protein database. Differences between HS1^T and HS5 homologs ≤ 1%.

Table 3. Homologs of *H. pylori* genes involved in induction of gastric lesions in the *H. suis* type strain 1 (HS1^T) and strain 5 (HS5) genome

Gene detected in HS1 ^T	Gene detected in HS5	Gene name	Protein annotation/function in <i>H. pylori</i>	Sequence fraction HS1 ^T /HS5 aligned with <i>H. pylori</i> homolog (%) ¹	Aligned sequence fraction HS1 ^T /HS5 conserved with <i>H. pylori</i> homolog (%) ¹	References
HSUHS1_0989	HSUHS5_0761	<i>vacA</i>	Vacuolating cytotoxin A: host cell vacuolation, apoptosis-inducing, immunosuppressive	63/22	45/72	(Gebert et al., 2004)
HSUHS1_0265	HSUHS5_0449	<i>ggt</i>	γ -glutamyl transpeptidase: apoptosis-inducing, immunosuppressive	99/99	86/86	(Shibayama et al., 2003; Kim et al., 2007; Schmees et al., 2007)
HSUHS1_1177	HSUHS5_0014	<i>napA</i>	Neutrophil-activating protein A: proinflammatory	99/99	83/83	(Brisslert et al., 2005; Wang et al., 2008)
HSUHS1_1067	HSUHS5_1177	<i>fldA</i>	Electron acceptor of the pyruvate oxidoreductase enzyme complex, associated with gastric MALT lymphoma in humans	96/98	84/83	(Chang et al., 1999; Freigang et al., 2001)
HSUHS1_0403	HSUHS5_0887	<i>pgbA</i>	Plasminogen-binding protein	60/60	72/72	(Ljung, 2000; Jönssen et al., 2004)
HSUHS1_1192	HSUHS5_0523	<i>pgbB</i>	Plasminogen-binding protein	70/70	72/72	(Ljung, 2000; Jönssen et al., 2004)

¹ Resulting from tblastn-based cross-mapping of the *H. pylori* proteome to the *H. suis* HS1^T and HS5 genomes.

Additional file

Additional file 1. Classification of *H. suis* strain 1 (HS1^T) and strain 5 (HS5) outer membrane proteins (OMPs) in relation to *H. pylori* OMPs

OMP family	Gene detected in HS1 ^T	Gene detected in HS5	Percentage of sequence fraction aligned (of which % conserved) with <i>H. pylori</i> homolog ¹
1. Hop-related OMPs			
HopE	HSUHS1_0015	HSUHS5_0838	99 (57)
HopG-2	HSUHS1_0180	HSUHS5_1092	92 (79)
HopH ²	HSUHS1_0340	HSUHS5_0513	83 (49)
HopW protein precursor	HSUHS1_1105	-	90 (71)
2. Hor-related OMPs			
HorA	-	HSUHS5_1252	100 (52)
HorB	HSUHS1_0666	HSUHS5_1053	100 (63)
HorC	HSUHS1_0472	HSUHS5_0483	99 (63)
HorD	HSUHS1_0202	HSUHS5_0115	91 (77)
HorE	HSUHS1_1144	-	96 (64)
HorF	-	HSUHS5_0133	85 (76)
HorJ	HSUHS1_0188	HSUHS5_0782	99 (53)
HorL	-	HSUHS5_0611	93 (67)
3. Hof-related OMPs			
HofA	HSUHS1_0120	HSUHS5_0671	93 (74)
HofC	HSUHS1_0181	HSUHS5_1091	99 (73)
HofE	HSUHS1_0179	HSUHS5_1093	98 (61)
HofF	HSUHS1_0178	HSUHS5_1094	95 (85)
Hof-family OMP	HSUHS1_0182	HSUHS5_1090	95 (84)
4. Iron-regulated OMPs			
Iron(III) dicitrate transport protein (FecA)	HSUHS1_0092	HSUHS5_0819	100 (89)
Iron(III) dicitrate transport protein (FecA)	HSUHS1_1124	HSUHS5_1010	96 (76)
Iron-regulated OMP (FrpB)	HSUHS1_1106	HSUHS5_0783	99 (79)
5. Efflux pump OMPs			
Omp of the hefABC efflux system (HefA)	HSUHS1_0643	HSUHS5_1029	82 (77)
Membrane fusion protein of the hefABC efflux system (HefB)	HSUHS1_0644	HSUHS5_1030	92 (79)
Cytoplasmic pump protein of the hefABC efflux system (HefC)	HSUHS1_0645	HSUHS5_1031	100 (78)

6. Unclassified OMPs³

Omp11 of <i>H. pylori</i>	-	HSUHS5_1235	99 (68)
Omp29 of <i>H. pylori</i>	HSUHS1_0008	HSUHS5_1251	94 (46)
Putative Omp of <i>H. pylori</i> (HPSH_03820)	HSUHS1_0646	HSUHS5_1032	100 (58)
Putative Omp of <i>H. pylori</i> (HP_1525)	HSUHS1_1135	HSUHS5_0620	94 (72)
Putative Omp of <i>H. pylori</i> (HPSH_03230)	HSUHS1_0446	HSUHS5_0930	96 (56)
Putative Omp of <i>H. pylori</i> (jhp_0368)	HSUHS1_0585	-	100 (67)
Putative Omp of <i>H. pylori</i> (jhp_0370)	HSUHS1_0583	-	95 (84)
Putative Omp of <i>H. pylori</i> (HP_1056)	HSUHS1_0584	-	74 (69)
Putative Omp of <i>H. pylori</i> (HPSH_02040)	-	HSUHS5_0158	75 (66)
Putative Omp of <i>H. pylori</i> (HPSH_02045)	-	HSUHS5_0156	95 (59)

¹ Resulting from tblastn-based cross-mapping of the *H. pylori* proteome to the *H. suis* HS1^T and HS5 genomes and blastp-based *ab initio* analyses of the translated *H. suis* HS1^T and HS5 ORFs against the Uniprot-KB universal protein database. Differences between HS1^T and HS5 homologs $\leq 1\%$. ² 99 amino acid HopH of *H. pylori* strain Shi 470 (HPSH_03675). ³ Predicted OMPs based on their N-terminal pattern of alternating hydrophobic amino acids similar to porins.

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CHAPTER 2

Immunization with the immunodominant *Helicobacter suis* urease subunit B induces partial protection against *H. suis* infection in a mouse model

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Abstract

Helicobacter (H.) suis is a porcine and human gastric pathogen. Previous studies in mice showed that a *H. suis* infection does not result in protective immunity, whereas immunization with *H. suis* whole-cell lysate (lysate) protects against a subsequent experimental infection. Therefore, two-dimensional gel electrophoresis of *H. suis* proteins was performed followed by immunoblotting with pooled sera from *H. suis*-infected mice or mice immunized with lysate. Weak reactivity against *H. suis* proteins was observed in post-infection sera. Sera from lysate-immunized mice, however, showed immunoreactivity against a total of 19 protein spots which were identified using LC-MS/MS. The *H. suis* urease subunit B (UreB) showed most pronounced reactivity against sera from lysate-immunized mice and was not detected with sera from infected mice. None of the pooled sera detected *H. suis* neutrophil-activating protein A (NapA). The protective efficacy of intranasal vaccination of BALB/c mice with *H. suis* UreB and NapA, both recombinantly expressed in *Escherichia coli* (rUreB and rNapA, respectively), was compared with that of *H. suis* lysate. All vaccines contained cholera toxin as adjuvant. Immunization of mice with rUreB and lysate induced a significant reduction of *H. suis* colonization compared to non-vaccinated *H. suis*-infected controls, whereas rNapA had no significant protective effect. Probably, a combination of local Th1 and Th17 responses, complemented by antibody responses play a role in the protective immunity against *H. suis* infections.

Introduction

Helicobacter (H.) suis is a world-wide spread pathogen, mainly colonizing pigs. An infection with this Gram-negative bacterium has been associated with ulcers of the gastric non-glandular mucosa (Roosendaal et al., 2000; Haesebrouck et al., 2009) and causes gastritis and decreased daily weight gain (De Bruyne et al., 2012) in pigs. *H. suis* is also the most prevalent non-*Helicobacter pylori* *Helicobacter* species in humans suffering from gastric disorders (Haesebrouck et al., 2009) and pigs may serve as a source of *H. suis* infections for humans (Meining et al., 1998; Haesebrouck et al., 2009). Control of *H. suis* infections by antibiotic-based therapy is not recommended partly due to an increased risk of developing acquired antimicrobial resistance in *H. suis* strains and in bacteria belonging to the normal porcine microbiota (Vermoote et al., 2011b). Immunization against *H. suis* may therefore represent a valuable alternative. Up to now, however, few studies have dealt with vaccination against this porcine and zoonotic pathogen.

Previous studies in a mouse model showed that a *H. suis* infection does not result in protective immunity, whereas vaccination based on homologous (*H. suis*) or heterologous (*H. bizzozeronii* or *H. cynogastricus*) whole-cell lysate induced a reduction or even complete clearance of gastric colonization with *H. suis* (Flahou et al., 2009). However, the use of this type of vaccines has drawbacks, including the laborious *in vitro* culture of *H. suis*, which results in difficulties to produce sufficient antigen. Also, whole-cell lysates may contain both protective antigens and antigens suppressing protection (Haesebrouck et al., 2004). An effective subunit vaccine might be a useful alternative for control of *H. suis* infections. Immunoproteomics is an appropriate approach for rapid identification of candidate proteins for vaccination and has been applied to study and develop subunit vaccines for a wide range of pathogens (Adamczyk-Poplawska et al., 2011).

It was the aim of the present study to select *H. suis* proteins which might induce protective immunity against *H. suis* infection. Therefore, *H. suis* proteins recognized by sera of mice immunized with *H. suis* whole-cell lysate and protected against infection were identified by using two-dimensional (2D) gel electrophoresis followed by immunoblotting and LC-MS/MS. Sera of *H. suis*-infected mice were also included, since an infection does not result in protection. Based on this analysis, the immunoreactive *H. suis* urease subunit B (UreB) was selected for further in

vivo testing. As a control we included the *H. suis* neutrophil-activating protein A (NapA), which has been previously described as a possible virulence factor (Vermoote et al., 2011a) but was not recognized by sera of mice immunized with whole-cell lysate. Subsequently, the protective efficacy against a *H. suis* infection of both subunit vaccines was evaluated and compared with that of *H. suis* lysate in a standardized mouse model.

Materials and methods

Bacterial strain

In all experiments, *H. suis* strain 5 (HS5, GenBank: ADHO000000000) was used. This strain was isolated from the gastric mucosa of a pig according to the method described by Baele et al. (2008).

Animals

One week prior to the initiation of the experiments, five-week-old specific-pathogen-free female BALB/c mice were obtained from an authorized breeder (HARLAN, Horst, The Netherlands). The animals were housed on sterilized wood shavings in filter top cages. They were fed with an autoclaved commercial diet (TEKLAD 2018S, HARLAN) and received autoclaved water *ad libitum*. All laboratory animal experiments were approved by the Animal Care and Ethics Committee of the Faculty of Veterinary Medicine, Ghent University.

Immunoproteomics of *H. suis*

Two-dimensional gel electrophoresis (2D-PAGE)

HS5 was grown as described previously (Flahou et al., 2010). Bacteria were harvested by centrifugation (5000 g, 4 °C for 10 min) and washed four times with Hank's balanced salt solution (HBSS). Total proteins (both soluble and insoluble proteins) were extracted in two steps using the ReadyPrep™ Sequential Extraction Kit (Bio-Rad, Hercules, CA, USA) according to manufacturer's instructions. In order to obtain good 2D-PAGE results, the homogenates were treated with proper additives (5 mg protease inhibitor cocktail, 1 µL DNase I, 1 µL RNase A, 10 µL phosphatase inhibitors PP2 and PP3 (Sigma-Aldrich, Steinheim, Germany)). Finally, the protein concentration was determined using the RC DC Protein Assay (Bio-Rad) and proteins were stored at -70 °C till further use. A total of 100 µg of HS5 proteins were rehydrated in

200 μ L rehydration buffer (7M ureum, 2M thiourem, 2% CHAPS, 0.2% carrier ampholyte pH3-4, 100mM dithiothreitol (DTT) and bromophenol blue). Samples were passively absorbed into a ReadyStrip (11 cm, pH3 to pH10, Bio-Rad) and iso-electric focusing was carried out in a Protean IEF Chamber (Bio-Rad) as previously described (Van Steendam et al., 2010). After iso-electric focusing, the strips were equilibrated for 15 min in 1.5% DTT in equilibration buffer (50mM TrisHCl, pH 8.8 6M urea, 20% glycerol, 2% SDS) followed by another equilibration in 4% iodoacetamide in equilibration buffer. Gel electrophoresis was carried out on a 10% TrisHCl SDS-PAGE using 150V for 30 min, followed by 200V for 1 h. Two gels were run in parallel: one was stained with Sypro[®] Ruby Protein Gel staining (Bio-Rad) while the other was used for immunoblotting (see Western blotting described below). Prior to staining, gels were fixed in 10% MeOH, 7% acetic acid. After staining, *H. suis* proteins were visualized using the VersaDoc Imaging System (Bio-Rad).

Serum pools

Three pools of mouse sera were used in this study:

- Sera from mice immunized with *H. suis* whole-cell lysate (hereafter referred to as “lysate-immunized mice”) ($n = 10$). These animals were inoculated intranasally twice with three weeks interval with 100 μ g HS5 lysate + 5 μ g cholera toxin (CT) (List Biological Laboratories Inc., Madison, NJ, USA). HS5 lysate was prepared as described previously but without final filtration of the supernatant (Flahou et al., 2009). Three weeks after the last immunization, blood was collected and sera were pooled. This immunization protocol has been shown to be (partially) protective against *H. suis* challenge (Flahou et al., 2009) and the protective effect was confirmed here in a preliminary experiment (data not shown).
- Sera from *H. suis*-infected mice (hereafter referred to as “infected mice”) ($n = 10$). These animals were inoculated intragastrically with 200 μ L Brucella broth at pH 5, containing 10^8 freshly prepared *H. suis* bacteria (Flahou et al., 2010). Four weeks after infection, blood was collected and sera were pooled.
- Sera from negative control mice ($n = 10$). These animals received HBSS intranasally twice with a three weeks interval followed by intragastric inoculation with 200 μ L Brucella broth at pH5 (4 weeks after last sham immunization). After four weeks, blood was collected and sera were pooled.

All sera were stored at -70 °C until further use.

Western blotting

Proteins were electrotransferred from gels onto nitrocellulose membranes (Bio-Rad) as described elsewhere (Van Steendam et al., 2010). Membranes were blocked in 5% skimmed milk in phosphate buffered saline (PBS) (blocking buffer), incubated overnight (ON) with diluted mouse sera (1/100 in blocking buffer) at room temperature (RT), rinsed in PBS with 0.3% Tween-20 (wash buffer) and incubated for 1 h at RT with stabilized goat anti-mouse immunoglobulin G (IgG) horseradish-peroxidase (HRP)-conjugated (1/1000 in blocking buffer, Pierce, Rockford, IL, USA). After a wash step in wash buffer, immunodetection of proteins was performed by enhanced chemiluminescence detection using Supersignal West Dura Extended Duration Substrate (Pierce). Protein patterns were scanned and digitized using the VersaDoc Imaging System. All experiments were performed in triplicate.

In-gel protein digestion and identification by mass spectrometry

In-gel digestion of proteins was performed as described by Cheung et al. (2009). Prior to mass spectrometry the isolated peptides were separated on a U3000 nano-high-performance liquid chromatography (HPLC) (Dionex, Sunnyvale, CA, USA) as previously described (Van Steendam et al., 2012).

Identification of the peptides was performed using an electrospray ionization quadrupole time-of-flight mass spectrometry (ESI-Q-TOF) Ultima (Waters, Milford, MA, USA) as described previously (Van Steendam et al., 2012). Data analysis was performed against the *Helicobacter* protein database from NCBI (146 612 entries) using the in-house search engine Mascot Daemon (2.3, Matrix Science, London, UK). An error tolerant search was performed with carbamidomethyl (C) as fixed modification. Carbamidomethyl (N-terminal) and oxidation (M) were set as variable modifications. Peptide mass tolerance and fragment mass tolerance was set at 0.35 Da and 0.45 Da, respectively. Maximum two miscleavages were allowed. Proteins were only considered to be correctly annotated when the significance was below 0.05 ($p < 0.05$) and at least one peptide passed the required bold red criteria from Mascot Daemon, indicating that at least one peptide had rank 1 and a significance below 0.05.

One-dimensional gel electrophoresis (1D-PAGE) and Western blotting of rUreB

1D-PAGE of 10 µg recombinant *H. suis* urease subunit B (rUreB) was performed as described by Van Steendam et al. (2010). Sera preparation and Western blot analyses were performed as described above.

Protective efficacy of recombinant *H. suis* proteins in a mouse model***Preparation of recombinant UreB***

A fragment encoding the *H. suis* UreB sequence (GenBank locus tag HSUHS5_0285) was amplified by PCR using a Pwo polymerase with proofreading activity (Roche, Mannheim, Germany) from the DNA of HS5 (forward primer: 5'- ATG AAA AAA ATC TCT AGG AAA GAA TAT G -3'; reverse primer: 5'- CTA GTG ATG GTG ATG GTG ATG GAA CAA GTT GTA GAG TTG AGC -3') and cloned into the protein expression vector pET-24d. The rUreB was expressed in *E. coli* strain BL21 (DE3). The cells were lysed by sonication (5 times for 30 s) in buffer containing 50mM Na₂PO₄ pH7, 0.5M NaCl, 1M DTT, 1% Triton X-100 and 1mM PMSF. After centrifugation (4 °C, 20 000 g for 30 min), rUreB was purified from the soluble fraction using Ni-affinity chromatography in buffer consisting of 1M NaCl, 50mM PBS, 1% Triton X-100, 250mM imidazole and 10% glycerol (His GraviTrap, GE Healthcare Bio-Sciences AB, Uppsala, Sweden) followed by gel filtration on a Superdex™ 200 HR 16/60 column (GE Healthcare Bio-Sciences AB). After purification, rUreB was analyzed using SDS-PAGE and Western blot analysis using anti-hexahistidine-tag mouse monoclonal antibody (Icosagen Cell Factory, Tartu, Estonia). The detergent Triton X-100 was removed from the purified rUreB by using Pierce Detergent Removal Spin columns (Pierce) following manufacturer's instructions. Protein concentration was determined with the RC DC protein Assay (Bio-Rad).

Preparation of recombinant NapA

The protein was expressed in the *E. coli* Expression System with Gateway® Technology (Invitrogen, Carlsbad, CA, USA) as follows. A fragment encoding the *H. suis* neutrophil-activating protein A (NapA) sequence (GenBank locus tag HSUHS5_0014) was amplified by PCR using a Pwo polymerase with proofreading activity (Roche) from the DNA of HS5 (forward primer: 5'- CACCATG AAAGCAAAAACAGTTGATGTACTC -3'; reverse primer: 5'- TTAAGCCAAACTTGCCTTAAGCATCC -3') and cloned into the pENTR™/TEV/D-TOPO® vector and transferred into the pDEST17™ destination vector. The selected pDEST17-NapA

plasmid was transformed to the BL21-AI™ *E. coli* and subsequently grown at 37 °C to an OD₆₀₀ of 0.6-1.0 in Luria Broth supplemented with 50 µg/mL carbenicillin. Recombinant *H. suis* NapA (rNapA) expression was induced by adding 0.2% L- arabinose. After 4 h incubation at 37 °C, the cells were harvested and resuspended in lysis buffer: 50mM TrisHCl, 100mM NaCl, 1% Triton X-100, 0.2 mg/mL lysozyme, 20 µg/mL DNase, 1mM protease inhibitor (Sigma), and 1mM MgCl₂. The cells were lysed by sonication (5 times for 30 s). Cell debris and inclusion bodies were isolated by centrifugation at 4 °C (20 000 g for 30 min). The inclusion bodies were subsequently washed twice based on the following protocol: the pellet was resuspended in cold lysis buffer, sonicated 5 times for 30 s followed by centrifugation (4 °C, 20 000 g for 30 min). The washed inclusion bodies were solubilized in binding buffer, pH8 (6M guanidium HCl, 20mM TrisHCl, 0.5M NaCl, 5mM imidazole, 1mM β-mercaptomethanol) by gentle rotation for 1 h at RT. Insoluble material was removed by high speed centrifugation at 4 °C (100 000 g for 30 min). rNapA was purified from the clarified supernatant onto a Ni-sepharose column (His GraviTrap, GE Healthcare Bio-Sciences AB) according to the manufacturer's instructions. rNapA was eluted with elution buffer, pH8 (8M urea, 20mM TrisHCl, 0.5M NaCl, 0.5M imidazole, and 1mM β-mercaptoethanol) and ON dialyzed against PBS at 4 °C. Afterwards, rNapA was analyzed using SDS-PAGE and protein concentration was determined using *RC DC* Protein Assay (Bio-Rad).

Immunization and infection experiments

The experimental design is summarized in Figure 1. Five groups of 10 mice were intranasally inoculated twice with 3 weeks interval, each time with 17.5 µL inoculum. In groups 1, 2 and 3 the inoculum consisted of HBSS with 5 µg CT, containing 30 µg rUreB, 30 µg rNapA and 100 µg HS5 lysate, respectively. Groups 4 (sham-immunized group) and 5 (negative control group) were inoculated with HBSS. Three weeks after the second intranasal immunization, blood was collected by tail bleeding from five animals per group and one week later, all animals, except the negative control group, were inoculated intragastrically with 200 µL Brucella broth at pH 5 containing 10⁸ viable *H. suis* bacteria (Flahou et al., 2010). The negative control group was inoculated intragastrically with 200 µL Brucella broth at pH5. Four weeks after the intragastric inoculation, mice were euthanized by cervical dislocation following isoflurane anaesthesia (IsoFlo; Abbott, IL, USA). From the euthanized animals, blood was collected by sterile cardiac puncture, centrifuged (1000 g, 4 °C, 10 min) and serum was frozen at -70 °C until further use.

Stomachs were excised and dissected along the greater curvature. One-half of the stomachs, including antrum and fundus, was immediately placed into 1 mL RNA Later (Ambion, Austin, TE, USA) and stored at -70 °C for further RNA- and DNA-extraction. A longitudinal strip of the gastric tissue was cut from the oesophagus to the duodenum along the greater curvature for histopathological examination.

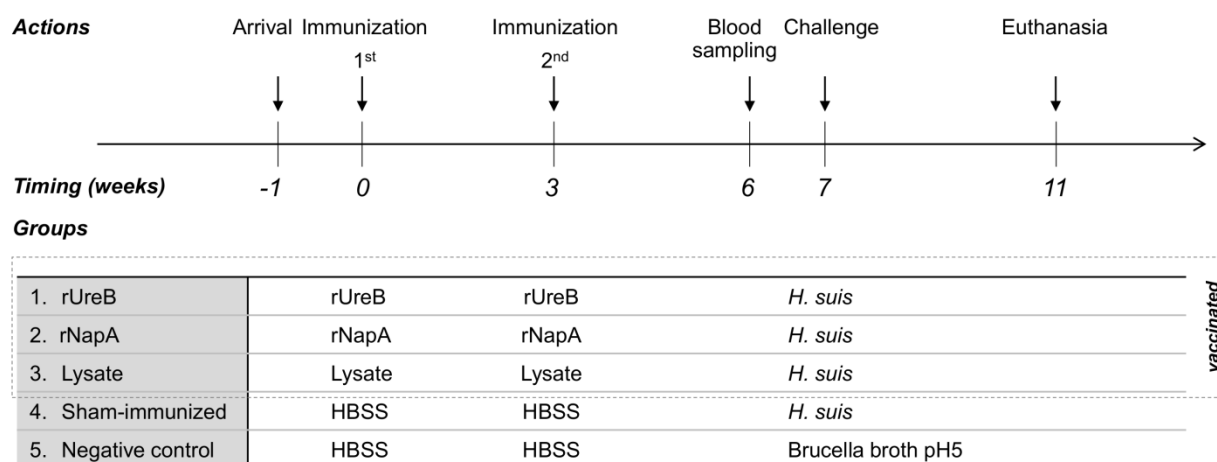


Figure 1. Experimental design of vaccination study. Per group 10 mice were intranasally immunized twice with 3 weeks interval, each time with 30 µg rUreB + 5 µg cholera toxin (CT); 30 µg rNapA + 5 µg CT, and 100 µg HS5 lysate + 5 µg CT (groups 1, 2 and 3, respectively). Groups 4 (sham-immunized group) and 5 (negative control group) were intranasally inoculated with HBSS. Three weeks after the second immunization, blood was collected from 5 mice per group and one week later mice of groups 1, 2, 3 and 4 were intragastrically inoculated with 10⁸ viable *H. suis* bacteria. Group 5 was intragastrically inoculated with 200 µL Brucella broth at pH5. Four weeks after intragastric challenge, mice were euthanized.

Quantification of *H. suis* in the stomach

After thawing, stomach tissues were homogenized (MagNAlyser, Roche, Mannheim, Germany) in 1 mL Tri Reagent[®] RT (MRC, Brunswick Chemie, Amsterdam, The Netherlands) and DNA was extracted from the inter- and organic phase according to Tri Reagent[®] RT manufacturer's instructions. The bacterial load in the stomach was determined using the previously described *H. suis* specific quantitative real-time PCR (qPCR) (Vermoote et al., 2011b).

Analysis of stomach cytokine response

The expression levels of IFN- γ , IL-4, IL-10, IL-17 and TNF- α were assessed by qPCR using cDNA synthesized from stomach tissue as described previously (Flahou et al., 2012). The threshold cycle (Ct) values were normalized to the geometric mean of the Ct-values from the reference genes after which normalized mRNA levels were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Measurement of serum antibody responses by enzyme-linked immunosorbent assay (ELISA)

The Protein Detector™ ELISA Kit (KPL, Gaithersburg, MD, USA) was used to evaluate rUreB-, rNapA-, and HS5 lysate specific IgG in serum. In brief, 96 well flat bottom plates (Nunc MaxiSorp, Nalge Nunc Int., Rochester, NY, USA) were coated with 2 μ g/well of purified rNapA, 1 μ g/well of purified rUreB, or 1 μ g/well of *H. suis* whole cell proteins diluted in 100 μ L coating buffer (24 h, 4 °C). After blocking with 1% bovine serum albumin in PBS, 100 μ L of 1/400 diluted serum was added to each well. After further washing, 100 μ L of HRP-labeled anti-mouse IgG (H+L) in a final concentration of 50 ng per well was added. Five minutes after adding 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) peroxidase substrate solution, absorbance was read at 405nm (OD_{405nm}).

Histopathological examination

The longitudinal gastric tissue strips were fixed in 4% phosphate buffered formaldehyde, processed by standard procedures and embedded in paraffin. For evaluation of gastritis, haematoxylin - eosin (HE) stained sections of 5 μ m were blindly scored based on the degree of infiltrating lymphocytes, plasma cells and neutrophils, using a visual analog scale similar to the Updated Sydney System (on a scale of 0-3) (Dixon et al., 1996) with the following specifications for each gastritis score: 0 = no infiltration of mononuclear and/or polymorphonuclear cells; 1 = mild diffuse infiltration of mononuclear and/or polymorphonuclear cells; 2 = moderate diffuse infiltration of mononuclear and/or polymorphonuclear cells and/or the presence of one or two inflammatory aggregates; 3 = marked diffuse infiltration of mononuclear and/or polymorphonuclear cells and/or the presence of at least three inflammatory aggregates.

Statistical analysis

Normality and variance homogeneity of data was analyzed by using D'Agostino-Pearson and Shapiro-Wilk normality test. Significant differences in *H. suis* colonization and mRNA cytokine expression among groups were assessed by performing one-way ANOVA analysis. Bonferroni's multiple comparison test was used as post-hoc when equal variances were assessed. Dunnett's T3 post-hoc test was used when no equal variances were assessed. OD_{405nm} levels from ELISA and histological inflammation scores were compared by Kruskal-Wallis analysis, followed by a Mann-Whitney *U* test. For correlations between different variables, Spearman's rho coefficient (ρ) was calculated. GraphPad Prism5 software (GraphPad Software Inc., San Diego, CA, USA) was used for all analyses. Statistically significant differences between groups were considered at $p < 0.05$.

Results**Immunoproteomics of *H. suis***

H. suis proteins were separated on 2D-PAGE (Figure 2a). After 2D-immunoblotting with pooled sera from lysate-immunized (Figure 2b) or *H. suis*-infected animals (Figure 2c), a total of 19 immunoreactive protein spots were selected. These spots were matched with the protein spots that could be seen in the parallel 2D-PAGE (Figure 2a). Little reactivity against *H. suis* proteins was observed in post-infection sera compared to the high reactivity against sera from lysate-immunized mice. When the blot was probed with a pool of sera obtained from negative control mice, no specific immunoreactive protein spots were detected (Additional file 1). Spots of interest ($n = 19$) were cut out of the gel, digested and identified by means of LC-MS/MS analysis. The detailed results of these proteins are summarized in Table 1. Spots with the highest reactivity (spot 1 to 5) were identified as UreB. *H. suis* chaperonin GroEL, illustrated as spots 9 and 10 on Figure 2a, showed also strong hybridization with sera from lysate-immunized animals. Additionally, sera from lysate-immunized mice showed strong reactivity against the urease accessory protein (UreH) and the urease subunit A (UreA) (spots 15 to 19), which was less pronounced in the infected group. Weak reactivity against the major flagellin FlaA (spots 11 to 13) was present in both blots.

Confirmation of serum reactivity against rUreB

From the 2D-analysis, UreB showed distinct reactivity with sera from lysate-immunized mice, which was not observed in sera from non-immunized but infected mice. In order to confirm these data, a 1D-PAGE loaded with rUreB was performed, followed by immunodetection with sera from lysate-immunized and *H. suis*-infected mice. Reactivity was only detected in the immunized group and a distinct band was visible at ~ 63 kDa, which corresponds to the molecular weight of UreB (Additional file 2).

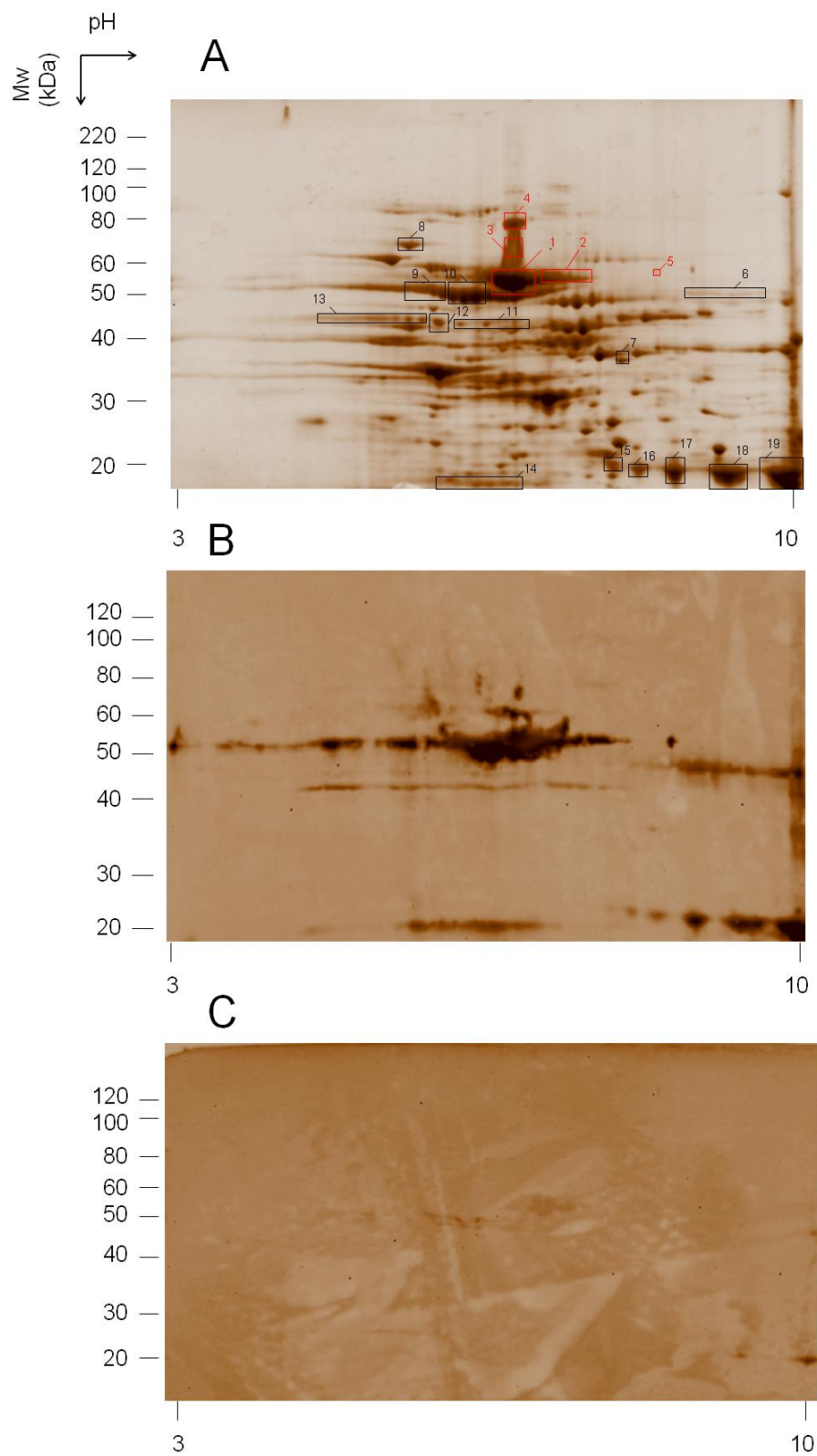


Figure 2. (see legend on next page)

Figure 2. *H. suis* 2D-proteome profile (A) and Western blots of a duplicate 2D-gel reacted with pooled sera of lysate-immunized mice (B) or of *H. suis*-infected mice (C). 100 µg of total protein extract of *H. suis* was separated by 2D-electrophoresis using linear pH3 to10 gradient in the first dimension and 10% TrisHCl SDS-PAGE in the second dimension. The separated proteins were detected by SYPRO®Ruby Protein staining. The boxed areas indicate where immunoreactive antigens were excised from the gel and subjected to LC-MS/MS. Identified proteins are indicated by the spot numbers given in Table 1. Boxes and numbers in red were identified as UreB. The position of molecular weight (MW) is given on the right, and the pH is given at the bottom.

Table 1. Immunoreactive proteins of *H. suis* identified by LC-MS/MS

Spot no. ¹	Protein name	NCBI ID ²	Gene	pI ³	MW ³ (kDa)	No. matched peptides	Mascot score ⁴	Cov. (%) ⁵
1	Urease subunit B	EFX42254	<i>ureB</i>	5.97	62.967	117	1589	72
2	Urease subunit B	EFX42254	<i>ureB</i>	5.97	62.967	80	1042	58
	Threonyl-tRNA synthetase	EFX41598	<i>thrS</i>	6.34	69.315	7	186	60
3	Urease subunit B	EFX42254	<i>ureB</i>	5.97	62.967	80	903	46
4	Urease subunit B	EFX42254	<i>ureB</i>	5.97	62.967	4	103	6
5	Urease subunit B	EFX42254	<i>ureB</i>	5.97	62.967	4	103	6
6	30S ribosomal protein S1	EFX42427	<i>rpsA</i>	8.29	64.051	23	625	28
	Quinone-reactive Ni/Fe hydrogenase, large subunit	EFX41851	<i>hydB</i>	8.22	64.943	19	520	28
	Urease of <i>H. heilmannii</i>	AAA65722		8.86	25.844	8	258	26
7	Methyl-accepting chemotaxis protein	EFX43528		7.1	48.907	35	905	50
8	Elongation factor G	EFX41637	<i>fusA</i>	5.15	77.242	57	1066	55
9	Chaperonin GroEL	EFX42237	<i>groEL</i>	5.58	58.498	150	3085	78
10	Chaperonin GroEL	EFX42237	<i>groEL</i>	5.58	58.498	135	2647	73
	Urease subunit B	EFX42254	<i>ureB</i>	5.97	62.967	43	682	41
11	Flagellin A	EFX41982	<i>flaA</i>	7.77	54.232	29	756	47
12	Flagellin A	EFX41982	<i>flaA</i>	7.77	54.232	57	1294	62
13	Flagellin A	EFX41982	<i>flaA</i>	7.77	54.232	46	1085	63
	Trigger factor	EFX42378	<i>tig</i>	5.13	49.587	12	343	24
14	Hydrogenase expression/formation protein	EFX41790	<i>hypB</i>	5.59	27.617	15	314	35
	Nicotinate-nucleotide pyrophosphorylase	EFX42191	<i>nadC</i>	6.46	30.591	11	219	25

	7-alpha-hydroxysteroid dehydrogenase	EFX41880	<i>hdhA</i>	6.62	28.246	6	186	24
	Conserved hypothetical secreted protein	EFX42511		5.84	28.011	5	174	19
	Hypothetical protein HSUHS5_0308	EFX42276		5.47	29.471	9	171	24
	Peroxiredoxin	EFX42277		5.84	25.811	7	107	19
15	Urease accessory protein	EFX42255	<i>ureH</i>	6.79	30.447	4	106	13
16	Urease subunit A	EFX42255	<i>ureA</i>	7.79	27.389	24	270	55
17	Urease subunit A	EFX42255	<i>ureA</i>	7.79	27.389	28	112	45
18	Urease subunit A	EFX42255	<i>ureA</i>	7.79	27.389	57	418	69
19	Urease subunit A	EFX42255	<i>ureA</i>	7.79	27.389	75	568	73

¹ Protein spot corresponding to position on gel and blots (see Figure 2).

² NCBI: National Center for Biotechnology Information.

³ Theoretical isoelectric point (pI) and molecular weight (MW).

⁴ For *Helicobacter* data, Mascot scores greater than 40 are significant ($p \leq 0.05$).

⁵ % of the protein sequence covered by the peptides identified.

Protective efficacy of recombinant *H. suis* proteins in a mouse model

From the 2D-proteomics approach, *H. suis* UreB was found to show a high reactivity with sera from lysate-immunized mice. Therefore, this protein was selected for further *in vivo* analyses. In addition, the *H. suis* NapA was tested. NapA has been previously described as a possible virulence factor of *H. suis* (Vermoote et al., 2011a), but was not detected by sera of lysate-immunized mice.

During the immunization experiment but before intragastric challenge, one animal from the rUreB immunized group and two animals from the group immunized with lysate died. The protective efficacy of rUreB, rNapA and lysate is shown in Figure 3 and expressed as the number of *H. suis* copies detected by qPCR in the stomach of challenged mice. High levels of *H. suis* ($> 10^5$ copies mg^{-1} stomach) were detected in the stomach of sham-immunized mice. Prophylactic immunization with rUreB induced a significant reduction in *H. suis* colonization compared to sham-immunized mice ($p < 0.001$). In contrast, immunization with rNapA did not induce a significant reduction ($p = 0.14$) in bacterial colonization. Immunization with lysate resulted in a significant reduction of the bacterial load ($p < 0.001$), and in 50% of the animals *H. suis* DNA was not detected by qPCR. A significant lower gastric bacterial load was observed in lysate-immunized mice compared to rUreB- and rNapA-immunized mice ($p < 0.01$).

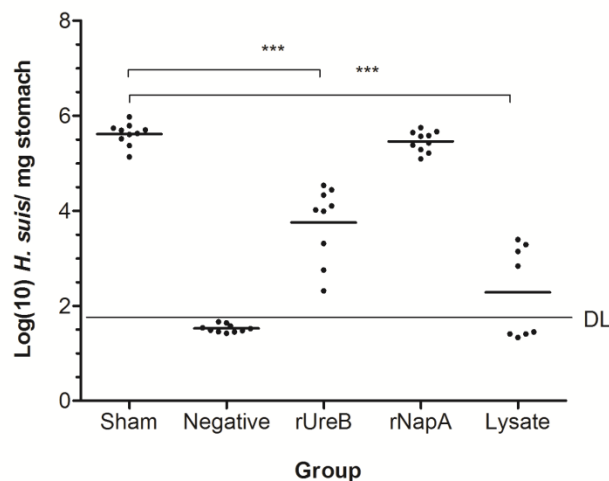


Figure 3. Protection against *H. suis* challenge after prophylactic intranasal immunization. Bacterial load is illustrated as log (10) of *H. suis* copies/mg stomach tissue. Individual mice are illustrated as dots around the mean (lines). DL: detection limit of 43.9 copies mg^{-1} . Significant differences between immunized (rUreB, rNapA and lysate) and sham-immunized, infected animals are noted by *** $p < 0.001$. Results of negative controls were all situated below DL.

Stomach cytokine response

mRNA expression levels of cytokines (IFN- γ , TNF- α , IL-4, IL-10, IL-17) in gastric tissue are illustrated in Figure 4. Expression of IL-17, a marker for a Th17 response, was increased ($p < 0.05$) in all immunized groups compared to sham-immunized mice. The IFN- γ response was significantly higher in rUreB- and lysate- immunized groups compared to the sham-immunized group ($p < 0.05$ and $p < 0.01$ respectively). Immunization with rNapA did not result in increased IFN- γ expression levels compared to sham-immunization ($p > 0.05$). When taking all groups inoculated intragastrically with *H. suis* into account (rNapA, rUreB, lysate and sham), a significant inverse correlation was observed between IL-17 and IFN- γ response on the one hand, and colonization on the other hand ($\rho = -0.388$ and $\rho = -0.816$, respectively, $p < 0.05$). IL-10 expression levels in the lysate-immunized group were significantly lower ($p < 0.05$) compared to all other groups (rUreB, rNapA and sham). A significant correlation was observed between IL-10 response and gastric colonization ($p < 0.01$, $\rho = 0.427$). IL-4 expression levels were higher in sham- and lysate-immunized groups compared to rNapA- and rUreB- immunized groups. This was significantly ($p < 0.05$) higher in lysate-immunized mice compared to rNapA-immunized mice. For TNF- α no significant differences in expression were observed between immunized and sham-immunized groups.

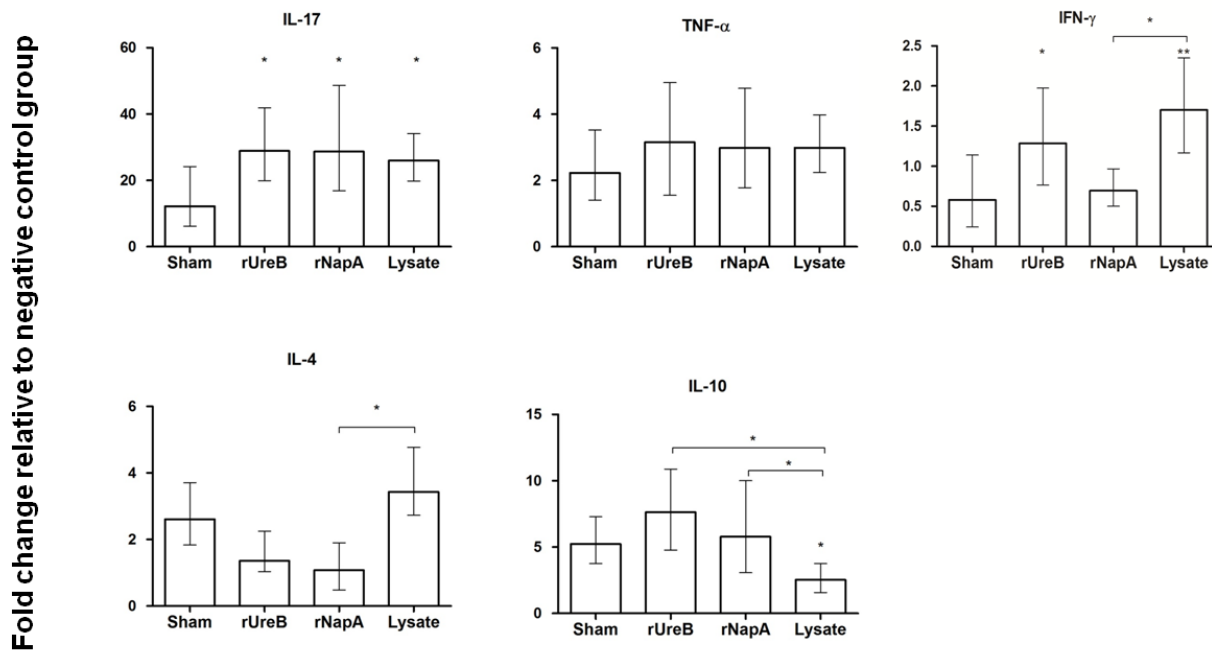


Figure 4. Fold change in cytokine gene expression level in the stomach relative to negative control animals. The stomach mRNA expression levels of cytokines (IL-4, IL-10, IL-17, IFN- γ and TNF- α) at final euthanasia were examined by qPCR. Data represent the normalized target gene amount relative to the negative control group which is considered 1. Data are shown as means \pm standard error of mean. Significant differences between immunized (rUreB, rNapA and lysate) and sham-immunized, infected animals are noted by * $p < 0.05$ and ** $p < 0.01$. Significant differences between immunized groups are noted by bars and * $p < 0.05$.

Specific serum antibody response before and after challenge

Three weeks after the last immunization and at euthanasia, serum was prepared for analysis of the serum-IgG response against rNapA, rUreB, and lysate. Serum levels of anti- rNapA, - rUreB and - lysate IgG of mice immunized with respective antigens were significantly elevated compared to negative controls at 3 week post-immunization (Additional file 3) and to both negative controls and sham-immunized mice at final euthanasia (Figure 5). Mice immunized with lysate showed a rUreB-specific serum IgG response but no rNapA-specific response (Figure 5b and c). When taking all immunized groups into account (rNapA, rUreB and lysate), a significant inverse

correlation ($\rho = -0.783$, $p < 0.001$) between specific IgG and *H. suis* copies mg^{-1} stomach was observed.

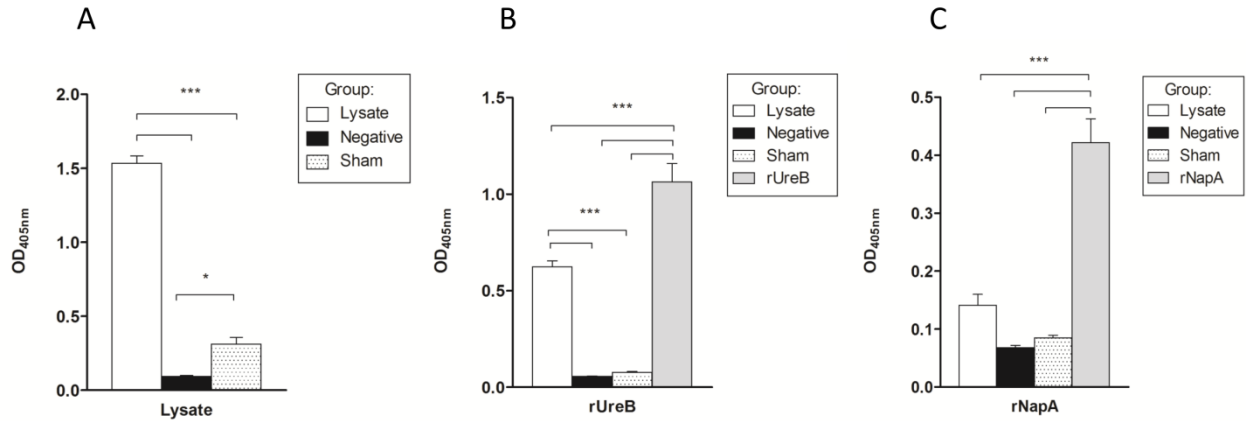


Figure 5. Serum antibody responses against lysate (A), rUreB (B) or rNapA (C) at euthanasia. The levels of specific IgG are shown as the mean OD_{405 nm} + SD. * $p < 0.05$, *** $p < 0.001$.

Histopathology

The overall gastric inflammation scores are presented in Table 2. All negative control mice showed normal histomorphology with very little inflammatory cell infiltration in the gastric mucosa. Sham-immunized mice developed a weak to moderate gastric inflammation. In general, higher inflammation scores were observed in the fundus compared to the antrum. Mice immunized with lysate showed a weak to moderate gastric inflammation, which was not significantly different from that observed in sham-immunized infected mice ($p > 0.3$). Although not significant ($p > 0.05$), less severe inflammatory infiltration was observed in rNapA and rUreB-immunized mice compared to mice immunized with lysate and sham-immunized mice.

Table 2. Gastric inflammation scores in mice after immunization and infection

Group	Inflammation score fundus ¹				Inflammation score antrum ¹				Overall mean inflammation score/group
	0	1	2	3	0	1	2	3	
rUreB	1	6	2	0	7	2	0	0	0.66
rNapA	4	6	0	0	8	2	0	0	0.40
Lysate	1	4	3	0	2	5	1	0	1.06
Sham-immunized	1	5	4	0	5	5	0	0	0.90
Negative control	10	0	0	0	10	0	0	0	0.00

¹Shown are the number of animals per group with a specific overall inflammation score in antrum and/or fundus: animals vaccinated with rUreB ($n = 9$), rNapA ($n = 10$) or lysate ($n = 8$), sham-immunized and infected controls ($n = 10$) and negative controls ($n = 10$). 0, no infiltration of mononuclear and/or polymorphonuclear cells; 1, mild diffuse infiltration of mononuclear and/or polymorphonuclear cells; 2, moderate diffuse infiltration of mononuclear and/or polymorphonuclear cells and/or the presence of one or two inflammatory aggregates; 3, marked diffuse infiltration of mononuclear and/or polymorphonuclear cells and/or the presence of at least three inflammatory aggregates.

Discussion

We previously demonstrated that immunization with *H. suis* whole-cell lysate protected mice against a subsequent experimental *H. suis* infection and resulted in high serum anti-*H. suis* IgG titers (Flahou et al., 2009). A *H. suis* infection, on the other hand, did not result in protective immunity, whereby significantly lower serum IgG titers were observed compared to *H. suis* protected animals (Flahou et al., 2009). In order to identify possible vaccine candidates, 2D-gel electrophoresis of *H. suis* proteins was performed followed by immunoblotting with pooled sera from *H. suis*-infected mice or mice immunized with *H. suis* whole-cell lysate. To our knowledge, this is the first study describing the immunoproteome of *H. suis*. The UreB protein showed a pronounced reactivity against sera from immunized mice and was not detected with sera from infected mice (Figure 2b and c). This protein was therefore selected for further evaluation of its protective efficacy. We found that immunization with rUreB resulted in a significant reduction of *H. suis* colonization in the stomach. The urease protein is known to be crucial for the survival of gastric *Helicobacter* species (Eaton et al., 1991; Haesebrouck et al., 2009) and vaccination with its subunit B (either natural or recombinant) also induced partial protection against *H. pylori*, *H. felis* and *H. heilmannii* (Ferrero et al., 1994; Michetti et al., 1994; Kleanthous et al., 1998; Dieterich et al., 1999; Bégué et al., 2010).

Vaccination with rUreB did not induce complete protection against an experimental *H. suis* infection. In contrast, a complete clearance was observed in 50% of mice immunized with whole-cell lysate, which is in line with previously observed results (Flahou et al., 2009). Most probably, in order to obtain a degree of protection which is similar to or better than that induced by whole-cell lysate, additional *H. suis* antigens will have to be included in subunit vaccines. The *H. suis* chaperonin GroEL (spots 9 and 10) is another protein that showed strong reactivity with sera from lysate-immunized mice and might therefore also be a candidate for inclusion in a subunit vaccine. Indeed, oral vaccination with *H. pylori* Hsp60 or *E. coli* GroEL induced a partial protection against *H. pylori* challenge (Ferrero et al., 1995; Yamaguchi et al., 2003). However, vaccination with this protein has also been associated with post-immunization gastritis (Yamaguchi et al., 2003). Additionally, it has been demonstrated that antibodies against *H. pylori* Hsp60 may be associated with gastric cancer and -inflammation in humans (Hayashi et al., 1998; Ishii et al., 2001; Tanaka et al., 2009). Other immunoreactive protein spots identified in this study

include UreA, UreH, FlaA, trigger factor, hydrogenase expression/formation protein, methyl-accepting chemotaxis protein and elongation factor G. All these proteins have also been identified in immunoproteomic studies of *H. pylori* and seem to be essential for gastric colonization of this bacterium (Kimmel et al., 2000). Future research is needed to determine the protective efficacy and possible side effects of vaccination with (combinations of) these proteins.

NapA has been recognized as a key modulator in *H. pylori*-induced gastritis (Brisslert et al., 2005) and has been proposed as a protective antigen and promising vaccine candidate against *H. pylori* infections (Satin et al., 2000; Malfertheiner et al., 2008). In the present study, NapA was not recognized by the pooled sera from lysate-immunized mice and intranasal immunization with rNapA did not result in protection against *H. suis* challenge, although it induced anti-rNapA IgG. The reason for the different outcome in protection studies with this protein in *H. suis* and *H. pylori* remains unclear. Differences in vaccine preparations, adjuvants and experimental infection models used may play a role. Although the *H. suis napA* gene shows strong homology with its *H. pylori* equivalent (99% of sequence aligned, of which 83% conserved) (Vermoote et al., 2011a), the role of NapA in the pathogenesis of *H. suis* infections has not yet been determined and is not necessarily identical to that of *H. pylori*.

Different immune mechanisms may be involved in protection induced by the vaccines tested here. Serum antibodies against rUreB or antigens present in *H. suis* lysate were detected in mice vaccinated with rUreB or lysate, respectively, while they were absent (rUreB) or remarkably lower in non-vaccinated, infected mice. In future studies it may be interesting to also determine IgA antibody titers locally produced in the stomach. The role of local and serum antibodies in protection against a *Helicobacter* infection is, however, controversial. Although several authors mentioned that they may play a role in protection (Czinn and Nedrud, 1991; Czinn et al., 1993; Blanchard et al., 1995; Lee et al., 1995; Ferrero et al., 1997; Jeremy et al., 2006), results of other studies indicate that prophylactic immunization against *Helicobacter* species does not require antibodies (Ermak et al., 1998; Blanchard et al., 1999). Whether circulating and/or local antibodies play a role in protection against *H. suis* infections may be determined by using antibody-deficient mice or by passive administration of serum antibodies (Czinn et al., 1993; Blanchard et al., 1995; Ermak et al., 1998; Blanchard et al., 1999; Keenan et al., 2000).

In mice vaccinated with rUreB or lysate, mRNA expression of IFN- γ , a signature Th1 cytokine, was significantly higher after challenge with *H. suis* compared to sham-immunized mice, and this was not demonstrated for rNapA-immunized, not protected mice. Moreover, a clear inverse correlation was observed between the bacterial load and IFN- γ mRNA expression levels. In non-vaccinated mice, a *H. suis* infection does not induce a Th1 response and does not result in clearance of the infection (Flahou et al., 2012). This indicates that production of IFN- γ , elicited by immunization, could play a role in suppression and clearance of *H. suis*.

Expression levels of IL-17 after challenge with *H. suis* were elevated in mice immunized with rUreB, rNapA and lysate, compared to sham-immunized mice and also for this cytokine, an inverse correlation with *H. suis* colonization was observed. In non-vaccinated mice, a *H. suis* infection mainly results in a Th17 response and a secondary Th2 response, which is not able to eradicate the infection although the Th17 response inversely correlates with bacterial load (Flahou et al., 2012). This might indicate that for a strong suppression or clearance of *H. suis*, a combined Th17 and Th1 response in the stomach is necessary, as was observed in the rUreB- and lysate-vaccinated groups.

We observed that decreased expression levels of IL-10 were correlated with a reduction in gastric *H. suis* colonization. This is not entirely unexpected, since IL-10 is a suppressive cytokine for Th17 and Th1. Additionally, it has been shown that IL-10-deficient mice are able to eradicate *H. pylori* infection (Matsumoto et al., 2005).

After infection with *H. suis*, expression of IL-4, a marker of a Th2 response, was higher in the lysate-immunized group than in groups vaccinated with rUreB and rNapA. Taken all results of the present study together, there are indications that in addition to a local Th1 and Th17 response, a Th2 response, probably resulting in local production of antibodies, may help to eradicate *H. suis* from the stomach. Indeed, only in the lysate-immunized group, mice were able to clear *H. suis* from the stomach. Further studies are, however, necessary to confirm this hypothesis.

In lysate-immunized mice, *H. suis* colonization was significantly lower than in the other experimentally infected groups. However, histological examination revealed that the inflammatory response in this group was almost similar to that in sham-immunized, *H. suis*-infected mice. For *H. pylori* too, a transient gastritis is often seen after challenge of immunized mice (Blanchard and Nedrud, 2010). It remains to be investigated whether gastritis levels of

lysate-immunized mice would drop below gastritis levels of sham-immunized animals after a longer period post challenge.

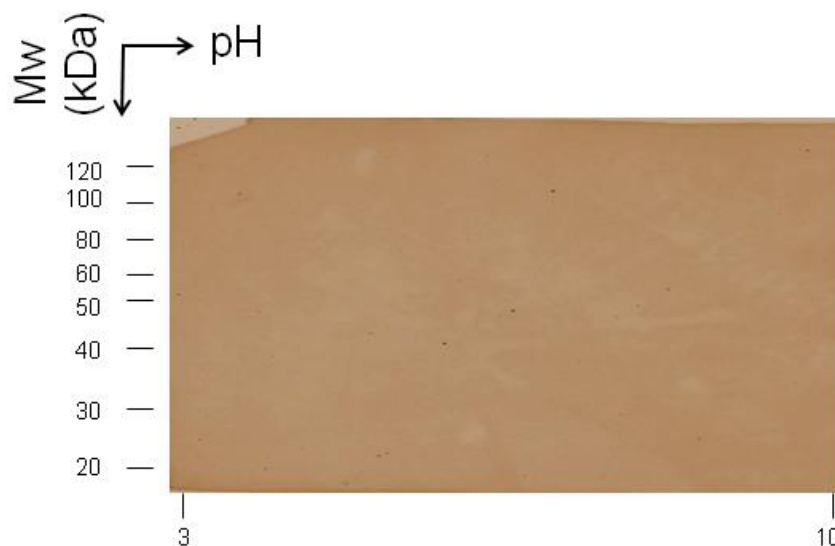
In conclusion, sera from lysate-immunized, protected mice strongly react with *H. suis* UreB and immunization with this antigen induced a significant reduction in gastric *H. suis* colonization in challenged mice. Although rUreB is a promising antigen candidate for the use in vaccines against *H. suis* infections, further studies are necessary to elucidate if inclusion of additional *H. suis* antigens may improve the protective efficacy of subunit vaccines. Also, results obtained in this mouse model should be confirmed in pigs, which are the natural host of *H. suis*. Probably, a combination of local Th1 and Th17 responses, complemented by antibody responses play a role in the protective immunity against *H. suis* infections. The exact mechanism by which protection against a *H. suis* infection is mediated remains however to be elucidated.

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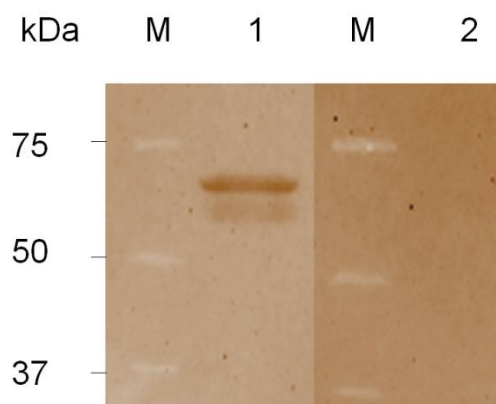
Additional files

Additional file 1. Immunodetection of a 2D-Western blot with a pool of control sera from *H. suis*-negative mice.



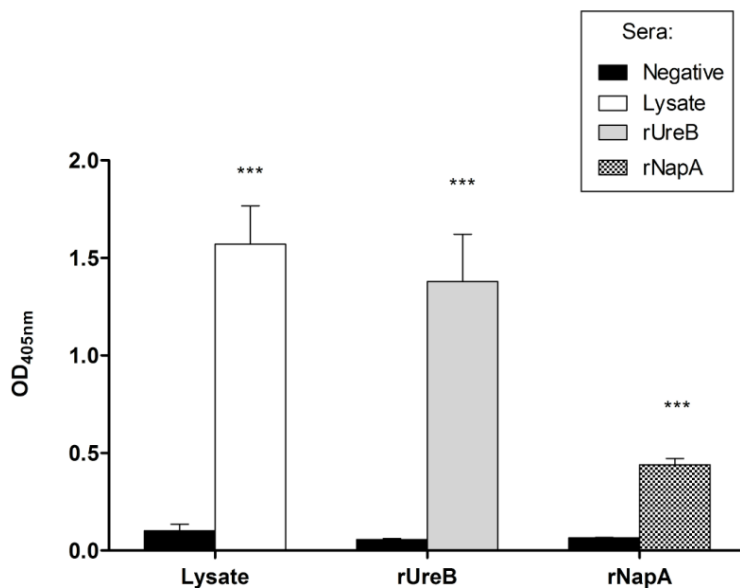
100 μ g of *H. suis* total protein extract was separated by 2D-electrophoresis using linear pH3 to10 gradient in the first dimension and 10% SDS-PAGE in the second dimension. After transfer of the proteins onto a nitrocellulose membrane, the 2D-immunoblot was analyzed by reacting with a pool of control sera from 10 *H. suis*-negative mice. No specific immunoreactive protein spots were detected.

Additional file 2. 1D-PAGE immunoblotting of rUreB.



M: Protein marker. Lane 1 and 2: 10 μ g rUreB separated on 10% TrisHCl SDS-PAGE and immunoblotted with serum of mice 3 weeks after immunization with *H. suis* whole-cell lysate (1) or with serum of *H. suis*-infected mice at four weeks post-infection (2). Both sera consisted of a pool of 10 animals. Only in serum of immunized animals (lane 1) immunoreactivity against rUreB is seen as a ~ 63 kDa band.

Additional file 3. Serum antibody responses against lysate, rUreB and rNapA at three weeks post-immunization.



Mice were immunized twice with three weeks interval with 100 µg HS5 lysate plus 5 µg CT, 30 µg rUreB plus 5 µg CT or 30 µg rNapA plus 5 µg CT, respectively. Three weeks after the last immunization blood was collected and serum was prepared from 5 animals of each group. Data are shown as the mean OD_{405 nm} + SD. *** $p < 0.001$.

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CHAPTER 3

Protective efficacy of vaccines based on the *Helicobacter suis* urease subunit B and γ -glutamyl transpeptidase

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Vaccine, provisionally accepted

Abstract

Helicobacter (H.) suis causes gastric lesions in pigs and humans. This study aimed to evaluate the protective efficacy of immunization with combinations of the *H. suis* urease subunit B (UreB) and γ -glutamyl transpeptidase (GGT), both recombinantly expressed in *Escherichia coli* (rUreB and rGGT, respectively). Mice were intranasally immunized with rUreB, rGGT or a combination of both proteins, administered simultaneously or sequentially. Control groups consisted of non-immunized and non-challenged mice (negative controls), sham-immunized and *H. suis*-challenged mice (sham-immunized controls), and finally, *H. suis* whole-cell lysate-immunized and *H. suis* challenged mice. Cholera toxin was used as mucosal adjuvant. All immunizations induced a significant reduction of gastric *H. suis* colonization, which was least pronounced in the groups immunized with rGGT and rUreB only. Consecutive immunization with rGGT followed by rUreB and immunization with the bivalent vaccine improved the protective efficacy compared to immunization with single proteins, with a complete clearance of infection observed in 50% of the animals. Immunization with whole-cell lysate induced a similar reduction of gastric bacterial colonization compared to rGGT and rUreB in combinations. Gastric lesions, however, were less pronounced in mice immunized with combinations of rUreB and rGGT compared to mice immunized with whole-cell lysate. In conclusion, vaccination with a combination of rGGT and rUreB protected mice against a subsequent *H. suis* infection and was not associated with severe post-vaccination gastric inflammation, indicating that it may be a promising method for control of *H. suis* infections.

Introduction

Helicobacter (H.) suis is a worldwide spread bacterium causing chronic gastritis and reduced daily weight gain in pigs (De Bruyne et al., 2012). An infection with *H. suis* has also been associated with erosive and ulcerative lesions in the non-glandular part of the porcine stomach (Barbosa et al., 1995; Rossesendaal et al., 2000). Furthermore, this bacterium is the most prevalent non-*H. pylori* *Helicobacter* species colonizing the stomach of humans suffering from gastric disease (Haesebrouck et al., 2009). Previous studies in mice have shown that prophylactic intranasal immunization with *H. suis* whole-cell lysate results in significant protection against *H. suis* infections (Flahou et al., 2009; Vermoote et al., 2012). However, production of sufficient *H. suis* whole-cell lysate may be hindered by the laborious *in vitro* cultivation of this bacterium. Also, whole-cell lysates may contain both protective antigens and antigens suppressing protection (Haesebrouck et al., 2004). To overcome these drawbacks, a subunit vaccine, based on the *H. suis* urease subunit B (UreB) has been developed (Vermoote et al., 2012). Immunization with *H. suis* UreB, recombinantly expressed in *E. coli* (rUreB) only induced a partial protection against *H. suis* challenge in a mouse model and it has been suggested that inclusion of additional antigens might improve the protective efficacy of this subunit vaccine (Vermoote et al., 2012).

In addition, immune modulating factors produced by the bacterium may hamper the development of a fully potent immune response against a *H. suis* infection, and thus may influence the effectiveness of certain vaccine formulations. Indeed, *H. suis* γ -glutamyl transpeptidase (GGT) has been shown to modulate the function of lymphocytes *in vitro*, which may result in host immune escape of *H. suis* leading to a chronic infection and lifelong persistence of *H. suis* in the porcine stomach (Zhang et al., unpublished data). Inhibition of this *H. suis* virulence factor by vaccination, may lead to an abrogation of its immune modulatory effect, enabling the development of a fully potent immune response against *H. suis* infection.

The aim of the present study was to evaluate the protective efficacy of simultaneous or consecutive immunization with recombinant *H. suis* GGT (rGGT) and rUreB against *H. suis* infections, and to compare it with that of *H. suis* lysate and univalent vaccination in a standardized mouse model.

Materials and methods

Bacterial strain

H. suis strain 5 was used in all experiments. This strain was isolated from the gastric mucosa of a sow, according to the method described by Baele et al. (2008).

Antigens for immunization

Recombinantly expressed GGT (rGGT) was prepared as described previously (Flahou et al., 2011). Briefly, HS5 DNA was used as template to PCR-amplify the *ggt* gene without predicted signal sequence, cloned into the pENTR™/SD/D-TOPO® vector and transferred into the pDEST™17 destination vector. Chemically competent *E. coli* BL21-AI™ cells were transformed and protein expression was induced with 0.2% L-arabinose. rGGT was purified by (His)₆-tag affinity on a Ni-sepharose column (His GraviTrap; GE Healthcare Bio-Sciences AB) following manufacturer's instructions. For further purification, the rGGT was loaded on a Superdex 75 gel filtration column (GE Healthcare Bio-sciences AB). Afterwards, rGGT was analyzed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and the GGT activity assay (Orlowski and Meiser, 1963).

Recombinantly expressed UreB (rUreB) was prepared as described previously (Vermoote et al., 2012). Briefly, a fragment encoding the HS5 UreB sequence was amplified by PCR and cloned into the protein expression vector pET-24d. The rUreB was expressed in *E. coli* strain BL21 (DE3) and *E. coli* cells were lysed by sonication in a buffer containing 50mM Na₂PO₄ pH7, 0.5M NaCl, 1M DTT, 1% Triton X-100 and 1mM PMSF. rUreB was purified using Ni-affinity chromatography in buffer consisting of 1 M NaCl, 50 mM PBS, 1% Triton X-100, 250 mM imidazole and 10% glycerol (His GraviTrap; GE Healthcare Bio-Sciences AB) followed by gel filtration on a Superdex™ 200 HR 16/60 column (GE Healthcare Bio-sciences AB). After purification, rUreB was analyzed using SDS-PAGE and Western-blot analysis using anti-hexahistidine-tag mouse monoclonal antibody (Icosagen Cell Factory, Tartu, Estonia). The detergent Triton X-100 was removed from the purified rUreB by using Pierce Detergent Removal Spin columns (Pierce Biotechnology, Rockford, USA) following manufacturer's instructions.

H. suis whole-cell lysate (lysate) was prepared as described by Flahou et al. (2009), but without final filtration of the supernatant. The latter was done to prevent potential loss of antigens.

Protein concentrations were determined with the *RC DC* protein Assay (Bio-Rad, Hercules, CA, USA).

Immunization and infection experiments

One week prior to the initiation of the experiments, 70 five-week-old specific-pathogen-free female BALB/c mice were obtained from an authorized breeder (HARLAN, Horst, The Netherlands). The animals were housed on autoclaved wood shavings in filter top cages. They were fed an autoclaved commercial diet (TEKLAD 2018S, HARLAN) and received autoclaved water *ad libitum*. All experiments involving animals were approved by the Animal Care and Ethics Committee of the Faculty of Veterinary Medicine, Ghent University (EC2011/164). Immunization and infection experiments were performed as presented in Figure 1. Mice were divided over seven groups of 10 animals each. Groups 1, 2, 3, 5, 6 and 7 were intranasally inoculated twice with three weeks interval, with a total volume of 17.5 μ l/ dose. In groups 1, 2, 3 and 5, vaccine formulations consisted of Hank's balanced salt solution (HBSS) with 5 μ g cholera toxin (CT) (List Biological Laboratories Inc., Madison, NJ, USA), to which 30 μ g rUreB, 30 μ g rGGT, 30 μ g rGGT + 30 μ g rUreB, and 100 μ g lysate had been added, respectively. Groups 6 (sham-immunized group) and 7 (negative control group) received HBSS only. Mice from group 4 were first immunized twice (with three weeks interval) with a vaccine consisting of HBSS, 5 μ g CT and 30 μ g rGGT. One week after the second immunization animals were immunized twice (with three weeks interval) with a vaccine consisting of HBSS, 5 μ g CT and 30 μ g rUreB. Three weeks after the last immunization, blood was collected by tail bleeding from five animals per group and one week later, all animals, except the negative control group, were intragastrically inoculated with 200 μ L Brucella broth at pH 5 containing 10^8 viable *H. suis* bacteria (Flahou et al., 2010). The negative control group was intragastrically inoculated with 200 μ L Brucella broth at pH 5. Four weeks after the challenge with *H. suis*, mice were euthanized by cervical dislocation following isoflurane anaesthesia (IsoFlo; Abbott, IL, USA). Blood was collected by sterile cardiac puncture, centrifuged (1000 g, 4°C, 10 min) and serum was frozen at -70°C until further use. Stomachs were excised and dissected along the greater curvature. One-half of the stomachs, including antrum and fundus, was immediately placed into 1 mL RNA Later (Ambion, Austin, TE, USA) and stored at -70°C for further RNA- and DNA-extraction. A longitudinal strip of gastric tissue was cut from the oesophagus to the duodenum along the greater curvature for histopathological examination.

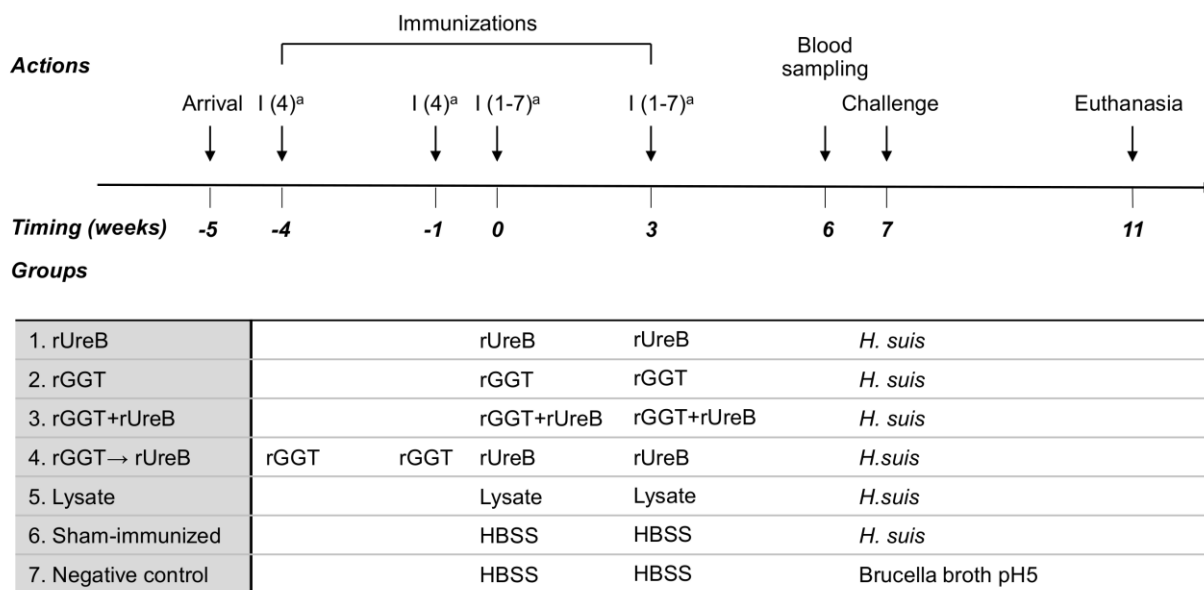


Figure 1. Experimental design of vaccination study. Per group 10 mice were intranasally immunized twice with 3 weeks interval, each time with 30 µg rUreB + 5 µg cholera toxin (CT), 30 µg rGGT + 5 µg CT, 30 µg rGGT + 30 µg rUreB + 5 µg CT, and 100 µg lysate + 5 µg CT (groups 1, 2, 3 and 5, respectively). Groups 6 (sham-immunized group) and 7 (negative control group) were intranasally immunized with HBSS. Mice from group 4 (rGGT→rUreB) were first intranasally immunized twice with 3 weeks interval, each time with 30 µg rGGT and 5 µg CT. One week after the second immunization animals were immunized twice with 3 weeks interval with 30 µg rUreB + 5 µg CT. Three weeks after the last immunization, blood was collected by tail bleeding from 5 animals per group and one week later, all animals, except the negative control group, were intragastrically inoculated 10^8 viable *H. suis* bacteria. The negative control group was intragastrically inoculated with 200 µL Brucella broth at pH5. Four weeks after challenge with *H. suis*, mice were euthanized. ^aI (x): Immunization of (number of group).

Determination of the number of *H. suis* in the stomach

After thawing, stomach tissues were homogenized (MagNAlyser, Roche, Mannheim, Germany) in 1 mL Tri Reagent[®] RT (MRC, Brunschwig Chemie, Amsterdam, The Netherlands) and DNA was extracted from the inter- and organic phase according to Tri Reagent[®] RT manufacturer's instructions. The bacterial load in the stomach was determined using a previously described *H. suis* specific quantitative real-time PCR (qPCR) (Vermoote et al., 2011).

Stomach cytokine responses

The mRNA expression levels of IFN- γ , TNF- α , IL-4, IL-10 and IL-17 were assessed by RT-qPCR using cDNA synthesized from stomach tissue as described previously (Flahou et al., 2012). The threshold cycle (Ct) values were normalized to the geometric mean of the Ct-values from the reference genes after which normalized mRNA levels were calculated using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001).

Serum antibody responses

Anti-rUreB, -rGGT and -lysate serum immunoglobulin G (IgG) responses were assessed by using the Protein Detector™ enzyme-linked immunosorbent assay (ELISA) Kit (KPL, Gaithersburg MD, USA). Measurement of anti-rUreB and -lysate specific serum IgG was performed as previously described (Vermootte et al., 2012). In brief, 96 well flat bottom plates (Nunc MaxiSorp, Nalge Nunc Int., Rochester, NY, USA) were coated with 1 μ g/well of purified rUreB, 2 μ g/well of purified rGGT, or 1 μ g/well of *H. suis* whole cell proteins diluted in 100 μ L coating buffer. After blocking with 1% bovine serum albumin in PBS, 100 μ L of 1/400 diluted serum was added to each well. After further washing, 100 μ L of HRP-labeled anti-mouse IgG (H+L) in a final concentration of 50 ng per well was added. Absorbance was read at 405nm (OD_{405nm}).

Histopathological examination

Longitudinal strips of gastric tissue were fixed in 4% phosphate buffered formaldehyde, processed by standard procedures and embedded in paraffin. For evaluation of gastritis, haematoxylin - eosin (HE) stained sections of 5 μ m were blindly scored based on the degree of infiltrating lymphocytes, plasma cells and neutrophils using a visual analog scale similar to the Updated Sydney System (on a scale of 0-3) (Dixon et al., 1996) with additional specifications for each score. The inflammation scores used in the grading system were as follows: 0, no infiltration with mononuclear and/or polymorphonuclear cells; 1, mild diffuse infiltration with mononuclear and/or polymorphonuclear cells; 2, moderate diffuse infiltration with mononuclear and/or polymorphonuclear cells and/or the presence of one or two inflammatory aggregates; 3, marked diffuse infiltration with mononuclear and/or polymorphonuclear cells and/or the presence of at least three inflammatory aggregates.

Statistical analysis

Significant differences in *H. suis* colonization and mRNA cytokine expression among groups were assessed by performing one-way ANOVA analysis. Bonferroni's multiple comparison test was used as post-hoc when equal variances were assessed. Dunnett's T3 post-hoc test was used when no equal variances were assessed. OD_{405nm} levels from ELISA were compared by Kruskal-Wallis analysis, followed by a Dunn's multiple comparison test. Histological inflammation scores were compared using the Mann-Whitney *U* test. For correlations between different variables, Spearman's rho coefficient (ρ) was calculated. GraphPad Prism5 software (GraphPad Software Inc., San Diego, CA) was used for all analyses. Statistically significant differences between groups were considered at $p < 0.05$.

Results

Protective effect of immunizations against *H. suis* challenge

All immunizations induced a significant reduction of gastric bacterial load compared to sham-immunized infected mice ($p < 0.05$), albeit significantly less pronounced in the group solely immunized with rGGT compared to all other immunizations ($p < 0.01$) (Figure 2). Highest levels of protection were seen in animals immunized with the combination of rGGT+rUreB or with lysate, with a 10 000-fold and 1000-fold reduction, respectively, of *H. suis* numbers (expressed as median) in the stomachs, compared to non-immunized infected controls ($p < 0.001$). Although not significant ($p > 0.05$), an enhanced protective effect was observed in mice immunized with combinations of rGGT and rUreB compared to rUreB alone. Immunization with rUreB alone, lysate, rGGT+rUreB and the subsequent immunization of rGGT followed by rUreB resulted in 33%, 50%, 57% and 44% of mice negative for *H. suis* DNA, respectively. Immunization with rGGT alone did not result in mice negative for *H. suis* DNA in the stomach. During the study 14 animals died, and the mortality rate per group is shown in Additional file 1.

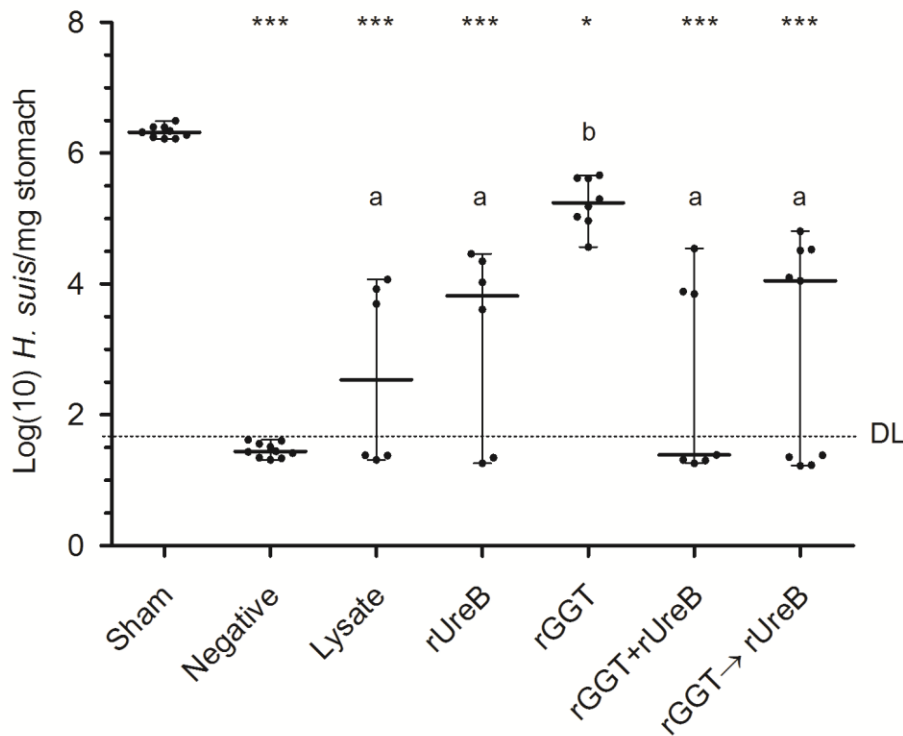


Figure 2. Protection against *H. suis* challenge after prophylactic immunization. Bacterial load per mg stomach tissue was determined for individual mice in each group by qPCR and is illustrated as dots with indication of median (horizontal lines) and range (vertical lines). The dotted line (DL) designates the detection limit of 41.8 copies/mg stomach tissue. * $p < 0.05$, *** $p < 0.001$ compared to non-immunized (sham) *H. suis*-challenged mice. Immunized groups which differed significantly ($p < 0.01$) are marked with different letters. rGGT→rUreB: group of mice which were sequentially immunized with rGGT and rUreB.

Stomach cytokine responses

mRNA cytokine expression levels (IFN- γ , TNF- α , IL-4, IL-10 and IL-17) in gastric tissue are presented in Figure 3. Significantly higher levels of IL-17 and INF- γ were observed in animals from all immunized groups, except in the group immunized with rGGT only, compared to sham-immunized mice ($p < 0.05$). Increased levels of IL-17 and IFN- γ were significantly correlated with a decrease in bacterial load ($p < 0.05$, $\rho = -0.513$ and $\rho = -0.2955$, respectively). For IL-4, IL-10 and TNF- α no significant differences in mRNA expression levels were seen between groups after infection. However, mRNA expression levels of TNF- α were increased in all immunized groups, except in the group immunized with rGGT only, compared to non-immunized mice. In addition, a mild negative correlation was observed between gastric bacterial load and

TNF- α expression levels ($p < 0.05$, $\rho = -0.349$). Lower levels of IL-10 were observed in immunized animals, compared to sham-immunized mice ($p > 0.05$) and a mild positive correlation was observed between gastric bacterial load and IL-10 expression levels ($p < 0.05$, $\rho = 0.356$).

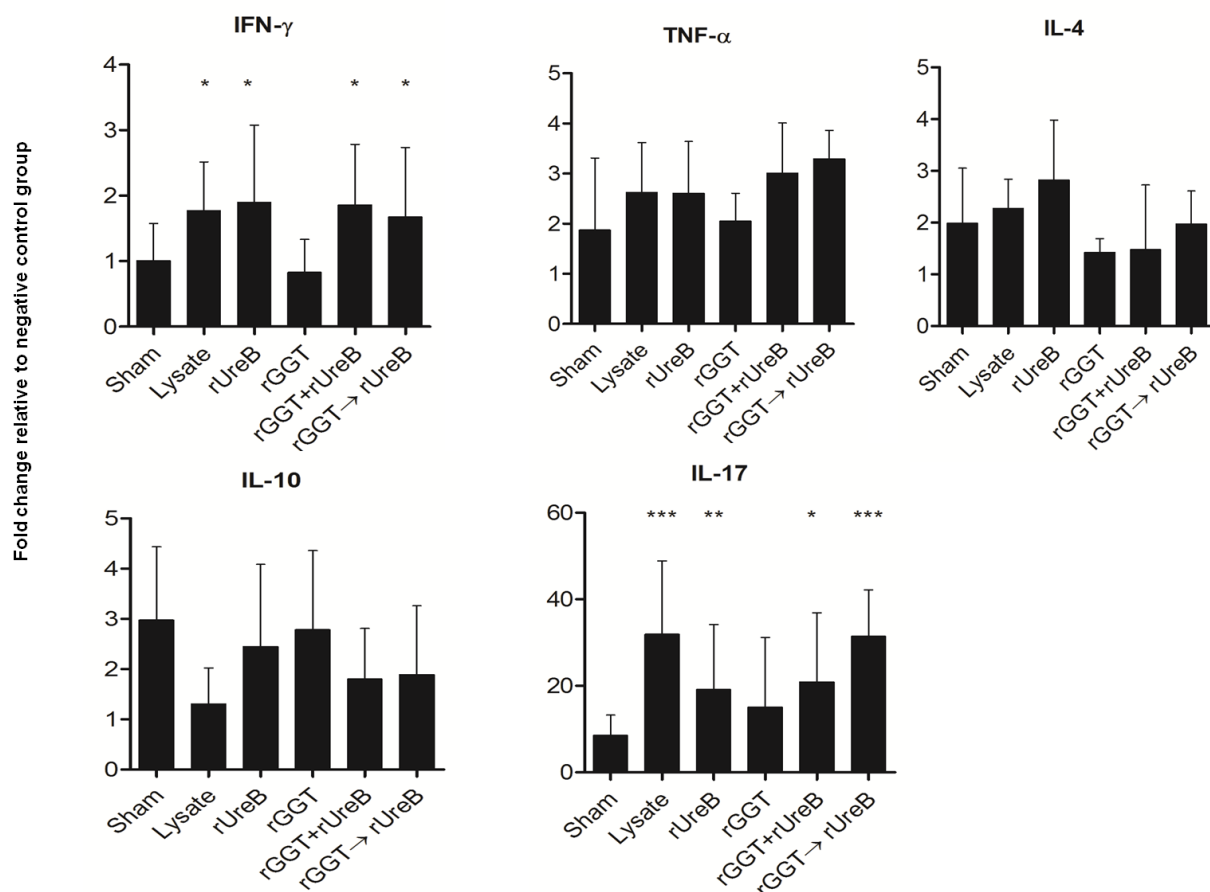


Figure 3. Fold change in cytokine gene expression levels in stomach tissue relative to negative control animals. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to non-immunized (sham) *H. suis*-challenged group. rGGT→rUreB: group of mice which were sequentially immunized with rGGT and rUreB.

Humoral immune responses

Three weeks after the last immunization, 1 week prior to challenge, specific serum anti-rUreB, anti-rGGT and anti-lysate IgG antibodies, were significantly increased in animals immunized with respective antigens compared to negative control mice (Additional file 2). Serum antibody

responses against *H. suis* lysate, rUreB and rGGT at euthanasia are shown in Figure 4. Negative controls and sham-immunized mice showed significantly lower anti-lysate, -rUreB and -rGGT serum IgG antibodies at euthanasia compared to groups vaccinated with lysate, -rUreB and/or -rGGT, respectively. A weak, but significant ($p < 0.05$, $\rho = -0.235$) correlation was observed between decreased bacterial load and increased specific serum IgG.

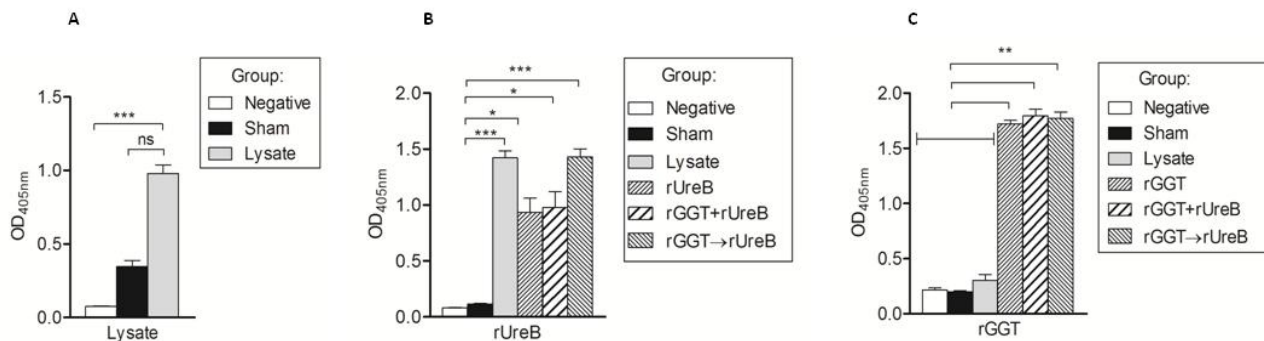


Figure 4. Serum antibody responses against *H. suis* lysate (A), rUreB (B) and rGGT (C) at euthanasia. Different groups are indicated by the bars with levels of specific IgG shown as the mean OD_{405nm} + SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns: not significant. rGGT→rUreB: group of mice which were sequentially immunized with rGGT and rUreB.

Histopathology

Figure 5 provides the results of histopathological examination of the stomachs. Higher inflammation scores were observed in the fundus compared to the antrum (Fig 5A and B). All negative controls had a normal gastric histomorphology (score 0) and sham-immunized, infected mice showed a weak gastric infiltration of mononuclear and/or polymorphonuclear cells (Fig 5C, median score of 0.25). Most pronounced inflammation was observed in mice immunized with *H. suis* lysate (Fig 5C, median score of 1.13), followed by animals immunized with rUreB alone (Fig 5C, median score of 1.00), mice immunized with the bivalent vaccine of rGGT and rUreB (Fig 5C, median score of 0.75). Immunization with rGGT alone and consecutive immunization of rGGT and rUreB resulted in less gastric infiltration of mononuclear and/or polymorphonuclear cells compared to all other immunizations, with a median score of 0.50 (Fig 5C). Inflammation in the fundic region and the average inflammation score of animals sequentially immunized with rGGT and rUreB were significantly lower compared to lysate-immunized mice ($p = 0.0033$ and $p = 0.0062$, respectively). Lysate-immunized mice also showed significantly higher overall gastric

inflammation and more severe inflammation in the fundic region compared to sham-immunized mice ($p = 0.016$ and $p = 0.013$, respectively). Average gastritis scores of *H. suis*-challenged groups immunized with lysate, rUreB, rGGT and rGGT+rUreB (simultaneously administered) differed significantly from that of non-infected negative control mice ($p < 0.05$).

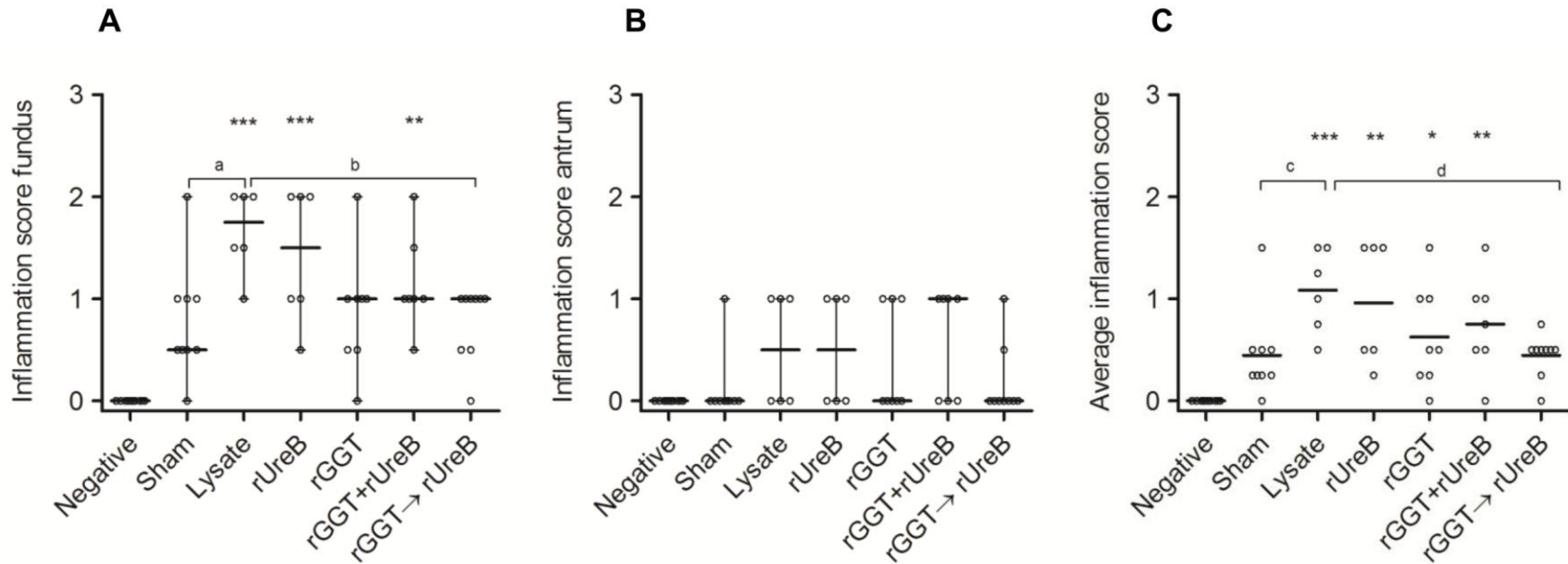


Figure 5. Gastric inflammation scores per group. Scores in negative controls, immunized and non-immunized (sham) mice 4 weeks after challenge were determined in fundus (A) and antrum (B) using haematoxylin-eosin-stained gastric sections. Average of inflammation score of fundus and antrum (average inflammation score) were calculated for each animal per group (C). 0, no infiltration with mononuclear and/or polymorphonuclear cells; 1, mild diffuse infiltration with mononuclear and/or polymorphonuclear cells and/or the presence of one or two inflammatory aggregates; 2, moderate diffuse infiltration with mononuclear and/or polymorphonuclear cells and/or the presence of at least three inflammatory aggregates; 3, marked diffuse infiltration with mononuclear and/or polymorphonuclear cells and/or the presence of at least three inflammatory aggregates. Gastric scores of individual mice per group are illustrated as dots with indication of median (horizontal lines) and range (vertical lines). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to non-infected negative control group. a: significant difference in inflammation in fundic region between sham-immunized and lysate-immunized mice, $p = 0.013$. b: significant difference in inflammation in fundic region between lysate-immunized mice and mice sequentially immunized with rGGT and rUreB (rGGT→rUreB), $p = 0.0033$. c: significant difference in average inflammation score between sham-immunized and lysate-immunized mice, $p = 0.016$. d: significant difference in average inflammation score between lysate-immunized mice and rGGT→rUreB immunized group, $p = 0.0062$.

Discussion

In a recent study, we demonstrated that intranasal vaccination with rUreB alone resulted in a significant reduction of gastric *H. suis* colonization, although complete protection was not achieved (Vermoote et al., 2012). Therefore, the present study aimed to evaluate whether a combination of rUreB and rGGT could increase the vaccine efficacy. *H. suis* GGT is a secreted virulence factor that acts in a similar way as the *H. pylori* GGT. The enzyme causes a glutathione degradation-dependent epithelial cell death (Shimayama et al., 2003; Flahou et al., 2011). In addition, it inhibits the proliferation of T-cells and thus may prevent the generation of an effective host immune response (Schmees et al., 2007; Zhang et al., unpublished data).

Although immunization with rUreB or rGGT alone induced a significant reduction of gastric *H. suis* colonization, consecutive or simultaneous immunization with both antigens was clearly more effective. The improved protective effect of vaccination with combinations of rGGT and rUreB compared to immunization with rUreB only, may be related to the consistent anti-GGT response, which might overcome immune evasion induced by this enzyme, enhancing clearance of the bacteria after challenge. Vaccination with *H. suis* rGGT alone seems, however, to be less effective than vaccination with rUreB alone.

H. suis colonization in mice generally induces a predominant Th17 response, in combination with a less pronounced Th2 response (Flahou et al., 2012). Despite this clear immune response, *H. suis* persists in infected mice. In contrast to *H. pylori* infection, *H. suis* infection does not result in increased levels of IFN- γ , a signature Th1 cytokine (Flahou et al., 2012). In the present study, vaccinated and protected mice showed significantly increased IFN- γ mRNA levels after challenge compared to sham-immunized mice and the degree of protection was correlated with increased levels of IFN- γ . Increased expression levels of the pro-inflammatory cytokine, TNF- α were also correlated with decreased bacterial gastric colonization. This indicates that a Th1 response may be involved in protective immunity against *H. suis* infection in mice. Indeed, we previously suggested that a combination of local Th17 and Th1 responses, complemented by antibody responses are involved in the protective immunity against *H. suis* infections (Vermoote et al., 2012). Also in this study the degree of protection was correlated with increased levels of IL-17, a marker of Th17 response, and specific serum IgG responses.

A decreased expression level of IL-10 was correlated with a reduction in gastric *H. suis* colonization. IL-10 is an anti-inflammatory cytokine, which is known to down-regulate immunity to infection and in this way may help gastric *Helicobacter* spp. to persist in their host (Matsumoto et al., 2005; Couper et al., 2008; Vermoote et al., 2012).

In addition to IL-10, combined vaccination of rGGT and rUreB depicts reduced levels of IL-4. Although increased levels of pro-inflammatory cytokines (IFN- γ , TNF- α and IL-17) and a downregulation of IL-10 and IL-4 mRNA were observed in mice immunized with combinations of rGGT and rUreB, rather a decrease of the microscopic gastric lesions were observed. The reason for this seemingly contradictory result remains to be investigated.

Ideally, an efficacious vaccine should induce protection whilst limiting side-effects. In prophylactic *H. pylori* mouse vaccination experiments, a more pronounced gastritis is often observed after challenging of immunized mice. This post-immunization gastritis is an important issue in the development of vaccines against *H. pylori*, especially when using whole-cell lysates (Goto et al., 1999; Garhart et al., 2002; Moihara et al., 2007). In the present study, the severity of gastric inflammation was higher in mice immunized with whole-cell lysate compared to other immunizations and sham-immunized, infected mice. Animals immunized first with rGGT followed by rUreB showed remarkably lower gastric inflammation compared to other immunized groups. The reason for this reduced inflammation is unclear and requires further studies. For example, the role of gastric and systemic cellular immune responses in induction and evolution of post-immunization gastritis may be determined by using CD4⁺-, B cell- or neutrophil deficient mice (Becher et al., 2010). In addition, it has been shown that post-immunization gastritis disappears over time, indicating that it is a transient event (Sutton et al., 2001; Garhart et al., 2002). A long term study could therefore be interesting to examine the evolution of the inflammatory response in all immunized, *H. suis*-challenged mice.

The results obtained in our mouse model may also be relevant for pigs, which act as the natural host of *H. suis*. Further studies are, however, necessary to confirm this. From an anatomical point-of-view, the nasopharynx-associated lymphoid tissue (NALT) of pigs is organized as tonsils, and forms the basis of the Waldeyer's ring (Liebler-Tenorio and Pabst, 2006), while NALT in rodents are presented as paired lymphoid aggregates in the floor of the nasal cavity at

the entrance to the pharyngeal duct (Kuper et al., 1992). In rodents, lymphocytes from the nose preferentially home back to NALT, as well as cervical and mesenteric lymph nodes, but not to Peyer's patches (Koornstra et al., 1992). It is not clear whether this is also true for lymphocytes of the porcine NALT. Nevertheless, intranasal vaccination of pigs could be a promising route of vaccination for inducing protection not only at the local mucosa, but also at distant mucosal surfaces, as has been described for immunization against enteric colibacillosis (Lin et al., 2013).

In this study, an unexpected high mortality was observed in immunized groups that were experimentally infected with *H. suis*, within days after challenge. This was not observed in sham-immunized and negative control groups, indicating that this most likely relates to the combination of immunization and subsequent challenge. The exact cause of death, however, was unclear. An extensive local immune response after immunization and subsequent challenge with *H. suis* might be a possible cause of death. Based on autopsy results of some animals, a pronounced local immune response related to the administration route (intranasal) and the adjuvant (CT) after immunization and subsequent challenge, may lead to excessive swelling of the nasal cavity mucosae, resulting in oxygen deficiency. In future *H. suis* mouse vaccination experiments, it should therefore be evaluated whether other mucosal administration routes, such as sublingual or oral immunization, could lead to a similar degree of protection without increased mortality.

In conclusion, immunization of mice with the combination of rGGT and rUreB, protected mice against a *H. suis* infection and induced less severe gastric lesions after *H. suis* challenge than immunization with a whole-cell vaccine. Both proteins are potential candidates for inclusion in subunit vaccines for control of *H. suis* infections. However, additional studies are needed to confirm the present results.

Acknowledgements

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Additional files

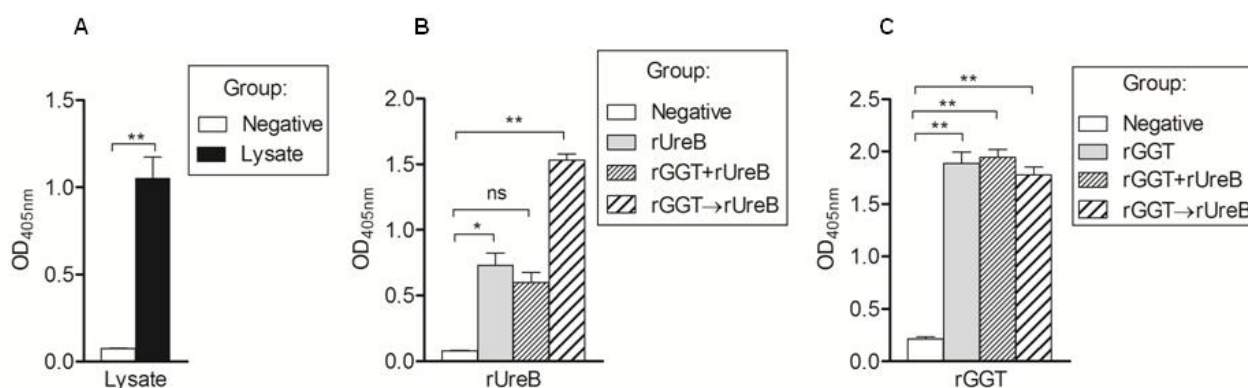
Additional file 1. Mortality rate in different groups during the study.

Group	Mortality rate (%)
1. rUreB	40% ^a
2. rGGT	20% ^a
3. rGGT+rUreB	30% ^a
4. rGGT→rUreB	10% ^a
5. Lysate	40% ^b
6. Sham-immunized	10% ^c
7. Negative control	0%

Mice of groups 1, 2, 3 and 5 were intranasally immunized twice with rUreB, rGGT, rGGT+rUreB or lysate, respectively. Groups 6 (sham-immunized group) and 7 (negative control group) were intranasally inoculated with HBSS. Mice from group 4 (rGGT→rUreB) were first intranasally immunized twice with 30 µg rGGT and 5 µg CT. One week after the second immunization animals were intranasally immunized twice with 30 µg rUreB and 5 µg CT. Four weeks after the last immunization, all animals, except the negative control group, were intragastrically inoculated 10^8 viable *H. suis* bacteria. The negative control group was intragastrically inoculated with 200 µL Brucella broth at pH5. Four weeks after challenge with *H. suis*, mice were euthanized.

^aMice died 2 to 5 days after intragastric challenge with *H. suis*. ^bTwo mice died before challenge: one animal 5 days after the first immunization and one animal 4 days after the second immunization. Two animals died 2 and 3 days after intragastric challenge with *H. suis*. ^cOne mouse was euthanized because of a reason unrelated to the study.

Additional file 2. Serum antibody responses against *H. suis* lysate (A), rUreB (B) and rGGT (C) at three weeks after last immunization.



Different groups are indicated by the bars with levels of specific IgG shown as the mean OD_{405nm} + SD. * $p < 0.05$, ** $p < 0.01$, ns: not significant. rGGT→rUreB: group of mice which were sequentially immunized with rGGT and rUreB.

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CHAPTER 4

Antimicrobial susceptibility pattern of *Helicobacter suis* strains

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Abstract

Helicobacter suis is a very fastidious porcine gastric pathogen, which is also considered to be of zoonotic importance. *In vitro* antimicrobial susceptibility can not be determined using standard assays, as this agent only grows in a biphasic medium with an acidic pH. Therefore, a combined agar and broth dilution method was used to analyse the activity of nine antimicrobial agents against nine *H. suis* isolates. After 48h microaerobic incubation, minimal inhibitory concentrations (MICs) were determined by software-assisted calculation of bacterial growth. Only for enrofloxacin a bimodal distribution of MICs was demonstrated, indicating acquired resistance in one strain, which showed an AGT → AGG (Ser → Arg) substitution at codon 99 of *gyrA*. In conclusion, the assay developed here is suitable for determination of the antimicrobial susceptibility of *H. suis* isolates, although activity of acid sensitive antimicrobial agents may be higher than predicted from MIC endpoints.

Introduction

Helicobacter suis is a large spiral-shaped bacterium that has been associated with chronic gastritis, ulcers of the gastric non-glandular mucosa (Haesebrouck et al., 2009) and decreased daily weight gain (Kumar et al., 2010) in pigs. It is also the most prevalent non-*Helicobacter pylori* *Helicobacter* (NHPH) species in humans suffering from gastric disorders (Van den Bulck et al., 2005a; Haesebrouck et al., 2009) and there are strong indications that pigs may act as a source of human infections (Meining et al., 1998). *H. suis* is a very fastidious microorganism requiring a highly enriched biphasic medium at pH 5 and a microaerobic atmosphere (Baele et al., 2008). Isolation of this agent from the gastric mucosa of pigs is a long and laborious process due to its slow growth and the risk of overgrowth with other bacteria which makes frequent subculturing necessary in order to obtain pure cultures containing sufficient numbers of *H. suis*. Therefore, it usually takes several weeks to obtain a single *H. suis* isolate.

Using a mouse model, it was shown that amoxicillin may be useful for treatment of *H. suis* infected animals (Hellemans et al., 2005). No other antimicrobial susceptibility data of *H. suis* have been published.

It was the aim of the present study to develop an *in vitro* assay allowing the determination of the antimicrobial susceptibility of *H. suis* isolates. This method was used to study the presence of acquired antimicrobial resistance in *H. suis* field isolates.

Materials and methods

Bacterial strains

Nine *H. suis* strains, isolated from the gastric mucosa of sows from different herds according to the method described by Baele et al. (2008), were included in this study. The microorganisms were grown under biphasic and microaerobic culture conditions at 37°C. The biphasic medium consisted of a Brucella agar (Oxoid, Basingstoke, England) supplemented with 20% fetal calf serum and Vitox supplement (Oxoid), with on top a Brucella broth (Oxoid). The pH of both agar and broth was adjusted to 5 by adding HCl. Isolates were passaged twice to ensure reliable growth. *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 29213, grown

overnight on Columbia agar (Oxoid) supplemented with 5% sheep blood, were included as reference strains.

Antimicrobial susceptibility testing of *H. suis*

Susceptibility to ampicillin, ceftiofur, clarithromycin, enrofloxacin, gentamicin, lincomycin, metronidazole, tetracycline and tylosin was investigated by a modified agar and broth dilution method in 24-well plates (Greiner Bio-On, Frickenhausen, Germany). The composition of the agar and broth were the same as described above, both with a pH of 5. All antimicrobials were supplied by Sigma (St. Louis, MO) as standard powders with known potencies, except for enrofloxacin, purchased from Bayer (Brussels, Belgium). The compounds were dissolved and diluted according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2008). Both agar plates and broth were freshly prepared and contained serial twofold dilutions of antimicrobial agents, with final concentrations ranging from 0.03 to 128 µg/ml. The broth contained 5×10^7 bacteria/ml of one of the *H. suis* strains. MICs for *H. suis* were determined after 48h microaerobic incubation by using quantitative real-time PCR (qPCR).

Suspensions with a density of 0.5 McFarland standard were prepared from overnight-grown reference strains *E. coli* and *S. aureus* and diluted to a concentration of approximately 5×10^5 bacteria/ml. For the reference strains two different MIC assays were performed: i) an assay in the biphasic and microaerobic conditions described for *H. suis* testing, and ii) the broth microdilution procedure according to the CLSI (CLSI, 2008). Wells containing agar and broth free of the tested antimicrobials served as controls. Plates were examined visually for the presence of bacterial growth, as indicated by turbidity after 24h for the reference strains.

All samples were tested in duplicate.

Quantitative real-time PCR

For *H. suis* quantification by qPCR, 10 fold dilutions ranging from 10^9 to 10^1 PCR amplicons of an external standard were included. This standard consisted of a 1116 bp segment of the *ureA* gene from *H. suis* and was constructed using ureaseA sense 5'- CGGGATTGATACCCACATTC and antisense 5'- ATGCCGTTTTTCATAAGCCAC primers. Amplicons were purified with Spin PCRapace (Invitek, Berlin, Germany) and dsDNA amount (ng/µl) was measured using a spectrophotometer. The copy number of the amplicon was calculated based on its dsDNA amount and length.

For enumeration of *H. suis* in the 24-well plates used for MIC determination, 100 µl aliquots of the broth were taken and DNA was isolated by using the PrepMan[®] Ultra Sample Preparation Reagent (Applied Biosystems, CA, USA), following the manufacturer's recommendations. DNA samples (1 µl) were analyzed in 9 µl amplification reactions consisting of 5 pmol of each qPCR primer (sense 5'-TTACCAAAAACACCGAAGCC, antisense 5'-CCAAGTGCGGGTAATCACTT), 3.1 µl distilled water and 5 µl iQ[™] SYBR[®] Green Supermix (Bio-Rad, Hercules, CA, USA). Thermal cycling (C1000[™] Thermal cycler, CFX96[™] Real Time system, Bio-Rad) consisted of an initial cycle of 95°C for 15min; 47 cycles of 95°C 20s, 60°C 30s, 73°C 30s followed by a melting curve analysis. In each qPCR analysis, negative-control reaction mixtures containing sterile water instead of DNA were included.

Both standards and samples were run in duplicate and the average values were used for quantification of *H. suis*. All samples were automatically processed for calculation of threshold cycles (Ct)-values and melting curve analysis of amplified DNA using the computer program Bio-Rad CFX Manager Version 1.1. (Bio-Rad). The Ct-value stands for the number of PCR cycles resulting in a positive quantitative test result.

The MIC for *H. suis* isolates in the present study was defined as the lowest concentration of an antimicrobial agent for which $\Delta Ct < 50\% \Delta cCt$, with $\Delta Ct = (Ct \text{ after incubation} - Ct \text{ before incubation})$ of antimicrobial-exposed sample, and $\Delta cCt = (Ct \text{ after incubation} - Ct \text{ before incubation})$ of controls. This is the lowest concentration of the antimicrobial agent with at least 50% less bacterial growth compared to controls without antimicrobials.

Sequence analysis of the quinolone-resistance determining region of gyrase genes

The quinolone-resistance determining regions (QRDRs) of *gyrA* (from codon 59 to 192) and *gyrB* (from codon 250 to 557) from *H. suis* strains HS1, HS5 and HS6 were amplified using a Pwo DNA polymerase (Roche, Mannheim, Germany) and sequenced, based on the conserved regions of *E. coli* (Yoshida et al., 1990; 1991) and *H. pylori* (Moore et al., 1995). The *gyrA* primers used were sense 5'-CCAGTACACCGGCGTATCCTTTATG and antisense 5'-CACAGCAATTCCGCTTGAGCCATTG, and the *gyrB* primers were sense 5'-TGGCTTTGGCTTATAATGAGG and antisense 5'-GCTATCAATGCCATGCTCAA. The sizes of the amplified fragments of *gyrA* and *gyrB* were 402 and 926 bp, respectively. Sequence analysis and alignments were performed using Vector NTI Software (Invitrogen, Carlsbad, CA, USA), and ClustalW.

Nucleotide sequences

Sequences of the *gyrA* gene in HS1, HS5 and the *gyrB* gene in HS1, HS5 have been deposited in the GenBank database with locus tags HSUHS1_1218, HSUHS5_0002 and HSUHS1_0588, HSUHS5_0161, respectively. The sequences of QRDR of *gyrA* and *gyrB* of HS6 are presented in Additional file 1.

Results

For the *E. coli* and *S. aureus* reference strains MIC endpoints of clarithromycin, gentamicin, lincomycin, and tylosin were respectively 10, 10, 14 and 4 times higher than the acceptable quality ranges of the CLSI when tested in the *H. suis* susceptibility assay conditions (CLSI, 2009). For the other tested antimicrobial agents they fell within acceptable quality ranges. The latter was also the case for all antimicrobial agents when tested in CLSI recommended conditions (CLSI, 2009).

MIC results for the *H. suis* isolates are summarized in Table 1. A monomodal distribution of MICs was seen for all antimicrobial agents, except for enrofloxacin for which a clear bimodal distribution was observed, indicating acquired resistance in one strain, HS6. An AGT → AGG (Ser → Arg) substitution at codon 99 of *gyrA* in this strain was identified by alignment of its *gyrA* QRDR sequence with the QRDR of strains HS1 and HS5 (MIC ≤ 0.06 µg/ml). No mutation was found in the *gyrB* QRDR.

Table 1. Distribution of MICs of nine *H. suis* isolates

Antimicrobial agent	No. of strains with a MIC (µg/ml) of:												
	≤0.03	0.06	0.125	0.25	0.5	1	2	4	8	16	32	64	128
Ampicillin					1	2	1	5					
Ceftiofur								1	5		3		
Clarithromycin			1	5	2	1							
Enrofloxacin	4	4					1						
Gentamicin								6	3				
Lincomycin								1	2	5		1	
Metronidazole								2	1	4	2		
Tetracycline				3	3	2	1						
Tylosin								4	5				

Discussion

The present study is the first to investigate the *in vitro* susceptibility of *H. suis* against antimicrobial agents. Currently, no standard methods have been described for MIC determination for NHPH species. MICs for *H. salomonis*, *H. bizzozeronii* and *H. felis* have been determined by using a slightly modified method (that is, horse blood instead of sheep blood) from that recommended for *H. pylori* and *Campylobacter jejuni* (Van den Bulck et al., 2005b). However, *H. suis* differs from other NHPH in its growth uniqueness. It does not form distinct colonies and needs specific biphasic culture conditions with an acidic pH. These growth characteristics impede agar dilution testing recommended by the CLSI (CLSI, 2008), leading to the use of a modified agar and broth dilution method in the present study.

Additionally, *H. suis* growth is not reliably detectable at visual inspection. Therefore, qPCR was used in this study as a valuable detection method for growth of these bacteria. qPCR has also been described to be a suitable alternative for *in vitro* susceptibility testing of other fastidious or intracellular bacteria, such as *Anaplasma phagocytophilum* (Hunfeld et al., 2004).

MICs of gentamicin, clarithromycin, tylosin and lincomycin were higher than the acceptable quality ranges for *E. coli* and *S. aureus* reference strains when tested in the *H. suis* susceptibility assay conditions. This may be due to the low pH of the media, necessary for *H. suis* growth, as activity of these antibiotics decreases at low pH (CLSI, 2009). Therefore, susceptibility of *H. suis*

to these antibiotics may be higher than predicted from the MIC endpoints detected here. Indeed, for *H. suis*, MICs of macrolides, lincomycin and gentamicin were higher than those described for *H. felis*, *H. bizzozeronii*, and *H. salomonis* (Van den Bulck et al., 2005b). On the other hand, in the present study pH conditions were not identical for the reference strains compared to the *H. suis* strains. In the medium of the *H. suis* strains without antimicrobials, a rapid increase from pH 5.35 to 7.00 was observed within 24h, which was not the case for the reference strains (data not shown). This is most probably due to the strong urease activity of *H. suis*.

A clear bimodal distribution of MIC values was demonstrated for enrofloxacin, indicating acquired resistance in one strain. In this strain, sequencing of the QRDR of *gyrA* showed a point mutation at position Ser99, which may be responsible for this resistance. In *H. pylori*, Asn87 and Asp91 point mutations in the QRDR have been associated with fluoroquinolone resistance (Moore et al., 1995).

Although the microbiological criterion used here for defining acquired resistance to enrofloxacin does not necessarily predict how a patient will respond to the therapy, the MIC value for the resistant strain was at least 30 times higher than for the susceptible population. The probability that humans or pigs infected with this isolate will respond well to treatment, should be considered to be low. The MICs of enrofloxacin for *H. suis* isolates without acquired resistance are similar to those described for *H. felis*, *H. bizzozeronii* and *H. salomonis* (Van den Bulck et al., 2005b).

For ampicillin, MICs are higher than those described for *H. felis*, *H. bizzozeronii*, *H. salomonis* and *H. pylori* (Loo et al., 1997; Van den Bulck et al., 2005b). This may indicate a reduced susceptibility of *H. suis* to ampicillin, compared with other gastric *Helicobacter* species.

In a previous study, *H. bizzozeronii* and *H. felis* isolates with MICs of metronidazole of 8-16 µg/ml were considered to be resistant to this antimicrobial agent (Van den Bulck et al., 2005b). In the present study, MICs of metronidazole showed a monomodal distribution but mostly fell in this higher MIC range. The significance of this finding for treatment of *H. suis* infections is not clear and it remains to be determined how a patient will respond to treatment with this antimicrobial agent.

MICs of tetracycline were in the range as described for *H. felis*, *H. bizzozeronii* and *H. salomonis* (Van den Bulck et al., 2005b). To our knowledge, MIC values of ceftiofur have not been published for other gastric NHPH species.

In conclusion, the assay developed in the present study is suitable for determination of the antimicrobial susceptibility of *H. suis* isolates, although activity of acid sensitive antimicrobial agents may be higher than predicted from MIC endpoints. Acquired resistance to fluoroquinolones may occur in *H. suis* field isolates. As the results of this study are based on a small number of strains, additional tests are needed to determine if these results reflect the susceptibility of the *H. suis* population.

Acknowledgments

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Additional file

Additional file 1. *Helicobacter suis* strain 6 quinolone-resistance determining region of *gyrA*.

```
1   CCAGTACACCGGCGTATCCTTTATGCTATGCACGAGTTAGGCTTGGGGG
50  CACGCGTGCCTTATAAAAAAAGTGCGCGCATTGTAGGCGATGTCATTGG
99  TAAATACCACCCCATGGCGATAGGGCAGTCTATGAGGCCTTAGTGCGC
148 ATGGCACAAGATTTTTCTATGCGTCTGCCTTTAGTAGATGGGCAAGGTAA
198 TTTTGGCTCTATTGATGGGGATAATGCCGCGGCGATGCGCTATACAGAGG
248 CACGCATGGCAACTCCAAGTGAGGAACTTTTAAGAGACATTGATAAAGAT
298 ACAGTAGATTTTAACGACAATTATGACAACACCCTAAAAGAACCCGATGT
348 CCTGCCTAGCCGTTTGCCTAATTTGCTCATCAATGGCTCAAGCGGAATTG
398 CTGTG
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GENERAL DISCUSSION

At the time this PhD was started, little was known about pathogenesis of *H. suis* infections and control strategies for this bacterium. This was mainly due to the lack of an *in vitro* isolation protocol for this very fastidious microorganism until 2008 (Baele et al., 2008). Availability of pure cultures of *H. suis* allowed in depth study of its genome and the identification of genes involved in bacterium-host interactions. It also facilitated evaluation of the usefulness of new control strategies against this microorganism.

1. New insights into the molecular pathogenesis of *H. suis* infections

H. pylori is considered the primary cause of human gastric disorders. However, in gastric biopsies of some patients with upper gastrointestinal symptoms, large, spiral-shaped non-*H. pylori* helicobacters (NHPH) are observed (Haesebrouck et al., 2009). In contrast to *H. pylori*, little research has been done on virulence and colonization mechanisms of NHPH in general and *H. suis* in particular. In **Chapter 1**, we performed a genome-wide analysis of two *H. suis* strains with the aim to obtain better insights into the colonization- and virulence factors of this bacterium.

H. suis is able to persistently colonize the stomach of pigs (Hellemans et al., 2007) and humans (Van den Bulck et al., 2005a; Joosten et al., 2013). Therefore, this bacterium needs to survive in the acidic environment, and to interact with the host using bacterial surface molecules (OMPs) that mediate adherence. Although comparative genome analysis of *H. suis* with *H. pylori* resulted in the detection of the vast majority of genes known to be essential for colonization of the human gastric mucosa, the *H. suis* genome lacks genes coding for BabA and SabA adhesins, which play a major role in *H. pylori* adhesion to the human gastric mucosa (Yamaoka and Alm, 2008). *H. suis* indeed differs from *H. pylori* in that it does not bind to the host cell-associated Le^b and sLe^x, two blood group antigens mediating the adherence of *H. pylori* by BabA and SabA, respectively (Mahdavi et al., 2002; Yamaoka, 2008). However, recent *in vitro* experiments demonstrated that *H. suis* could adhere to host mucins (Smet A. and Lindén S., personal communications, 2012), which indicates that adherence to host mucins may play a role in the initial colonization and long-term persistence of *H. suis* in the stomach, and that it is mediated by adhesins other than BabA and SabA. Possible candidate adhesins of *H. suis* are the HpaA, HorB and Hof proteins. Genes encoding these OMPs were detected in the sequenced *H. suis* genomes and are also present in the genomes of other sequenced NHPH (Arnold et al., 2011; Schott et al., 2011; Smet et al., 2013).

Future studies, including adhesion experiments with *H. suis* mutant strains, should elucidate the exact role of these OMPs in gastric colonization by these bacteria.

Before our study, virtually nothing was known about possible *H. suis* virulence factors involved in gastric pathology. As described in the general introduction of this thesis, VacA and CagA play a key role in pathogenesis of *H. pylori* infections (Delahay and Rugge, 2012). VacA induces vacuolization and apoptosis in epithelial cells (Cover and Blaser, 1992; Cover et al., 2003), while CagA, as a member of the *cag* pathogenicity island, is responsible for cytoskeletal changes and production of pro-inflammatory NF- κ B (Mimuro et al., 2002; Brandt et al., 2005). Genes encoding a functional VacA and CagA were neither found in *H. suis*, nor in other gastric *Helicobacter* species (Dailidienė et al., 2004; O'Toole et al., 2010; Arnold et al., 2011; Schott et al., 2011; Smet et al., 2013). Despite the absence of these major *H. pylori* virulence factors, infections with NHPH species have been associated with gastric lesions. For instance, *H. suis* and *H. felis* induce extensive apoptosis and necrosis of parietal cells in experimentally infected mice and/or gerbils (De Bock et al., 2006; Flahou et al., 2010), which may have important implications for the development of gastric pathologies, such as gastric ulcer formation (Dixon, 2001), gastric atrophy and gastric cancer (Shirin and Moss, 1998). Apart from VacA it has been shown that the *H. pylori* γ -glutamyl transpeptidase (GGT) may induce gastric epithelial cell death (Shibayama et al., 2003; Gong et al., 2010). In the *H. suis* genome, we found a homolog of the *H. pylori* GGT-coding gene, and proposed it as a potential virulence factor of *H. suis*. Recent work indeed showed that the *H. suis* GGT is involved in the induction of gastric lesions (Flahou et al., 2011). The authors showed that this enzyme is largely responsible for the induction of apoptosis and necrosis of AGS cells by *H. suis* (Flahou et al., 2011).

Although the incidence of NHPH gastritis is considered lower than that of *H. pylori*, the risk of developing gastric MALT lymphoma is higher in NHPH-infected patients compared to those infected with *H. pylori*. A long-term study of *Helicobacter*-related gastric pathology indeed showed that 1.48% and 0.66% of the patients infected with NHPH and *H. pylori*, respectively developed MALT lymphoma (Stolte et al., 1997; 2002). The development of MALT lymphoma has been associated with a Th2 response, rather than with a Th1-predominant response (Knörr et al., 1999). In contrast to the predominant Th17/Th1 response evoked by a *H. pylori* infection (Robinson and Atherton, 2010), *H. suis* infections have been shown to induce a Th17/Th2 response in mice (Flahou et al., 2012). Interestingly, *H. pylori* harbors some virulence factors that

modulate the host immune response toward a Th1 response, including the *cag* pathogenicity island; the outer inflammatory protein A, OipA; and the neutrophil-activating protein, HP-NAP (Amedei et al., 2006; D'Elia et al., 2007; Yamauchi et al., 2008). Except for HP-NAP, *H. suis* lacks homologs of these factors, which could possibly explain the absence of a Th1 response associated with a *H. suis* infection in mice, and the higher incidence to develop MALT lymphoma compared to *H. pylori* infections. In addition, a homolog of the flavodoxin A (FldA)-coding gene has been found in the *H. suis* genome. The *H. pylori* FldA is associated with MALT lymphomas in humans (Chang et al., 1999). The exact role of HP-NAP and FldA as putative *H. suis* virulence factors in gastric diseases remains to be elucidated.

Another point to consider is the role of *Helicobacter* virulence factors in host immune evasion, which may result in a persistent infection. The *H. pylori* VacA and GGT have been shown to inhibit T cell proliferation (Sundrud et al., 2004; Schmees et al., 2007). As discussed above, only a homolog for the GGT-coding gene is present in the *H. suis* genome. Very recently, the *H. suis* GGT has been described to impair proliferation of Jurkat T cells as well as different subsets of primary mouse lymphocytes, and both cell death and cell cycle arrest seem to be involved (Zhang et al., unpublished data). Experiments using *ggf* *H. suis*-mutants and subsequent evaluation in rodent models will clarify the exact mechanism of *H. suis* GGT-induced immune modulation.

2. New insights into proteins inducing a protective immune response against *H. suis*

H. suis infections have been described in more than half of the world's pig population (Haesebrouck et al., 2009). Antimicrobial therapy in pigs is therefore an impractical approach to control *H. suis* infections. In addition, antimicrobial-based therapy in pigs is not recommended partly due to increased human health risks associated with antimicrobial use in food producing animals, including the spread of antimicrobial resistance in pathogens and in bacteria belonging to the normal microbiota (Jensen et al., 2008). Vaccination of pigs may therefore represent a valuable alternative to control *H. suis* infections in pigs and humans.

Different approaches can be used to select potential candidate vaccine antigens. In **Chapter 2**, the immunoproteomics approach was used to select a first vaccine candidate, the urease subunit B (UreB). A second candidate, the *H. suis* GGT, was selected based on its importance in *H. suis*-induced gastric pathology and immunoregulatory capacities (Chapter 3).

In addition to insights into genes involved in gastric pathology, genome analysis can be used to identify potentially immunogenic proteins by examining their predicted subcellular localization (membrane or secreted proteins) or by homology with virulence factors of related pathogens (Serruto et al., 2009). In the first study of this thesis, we determined approximately 95 and 208 open reading frames with a predicted signal peptide cleavage site and transmembrane helix, referring to secreted proteins and membrane-associated proteins, respectively. However, to reduce this huge haystack of ~ 303 possible antigen candidates, additional tools providing more information about host-bacterium relationship and immunogenicity of the proteins were needed. Proteomics is particularly useful as it directly reveals actual antigenic properties such as abundance, localization and seroreactivity, instead of indirect data as provided by genome-based techniques (Bumann et al., 2004). In order to identify potential vaccine candidates, we performed two-dimensional gel electrophoresis of *H. suis* proteins, followed by immunoblotting with sera from vaccinated and protected mice on the one hand, and sera from *H. suis*-infected and non-protected mice on the other hand. To our knowledge, this is the first study describing the immunoproteome of *H. suis*. This approach has already been used to identify new *H. pylori* antigens of protective value (McAtee et al., 1998; Hocking et al., 1999; Bumann et al., 2002; 2004). In our experiments, four major proteins of protective interest could be identified with sera from *H. suis* lysate-immunized mice, being UreB, chaperonin GroEL, FlaA and UreA. These proteins have also been identified in several *H. pylori* immunoproteome studies when using sera from *H. pylori*-infected humans (McAtee et al., 1998; Hocking et al., 1999; Kimmel et al., 2000; Bumann et al., 2002; Lock et al., 2002), and showed to be of protective value against *H. pylori* infections in mice (Skene et al., 2007; Blanchard and Nedrud, 2010). The abundance of UreB and GroEL could possibly obscure the detection of low-abundant proteins. Low-abundant proteins may also be poorly recognized by the host immune system as antigen recognition is generally dose-dependent. This does, however, not exclude their possible role in protection if included in vaccines. Experiments in which *H. suis* whole-cell proteins are fractionated based on molecular weight (size) may allow discovery of these proteins.

The few vaccination studies that had been carried out for *H. suis* only focused on the use of whole-cell lysates of homologous or heterologous bacteria (Hellemans et al., 2006; Flahou et al., 2009). Despite positive results obtained during these studies, safety and practical considerations related to the large-scale culture of *H. suis in vitro* are substantial obstacles in developing a *H.*

suis whole-cell vaccine for pigs and/or humans. In **Chapters 2 and 3** we evaluated the protective efficacy of a limited number of subunit vaccine candidates, and compared it with that of whole-cell lysate in a mouse model. In a first study, we developed a subunit vaccine based on *H. suis* UreB, recombinantly expressed in *E. coli* (rUreB) (Chapter 2). Intranasal immunization with rUreB and cholera toxin (CT) resulted in a significant decrease of the bacterial load in the gastric mucosa, but could not prevent *Helicobacter* colonization. Immunization with whole-cell lysate, however, induced higher levels of protection compared to immunization with rUreB. Therefore, in a subsequent study, we investigated the usefulness of rUreB in combination with another *H. suis* protein. Simultaneous and consecutive immunization with the recombinant *H. suis* GGT (rGGT) and rUreB induced better protection against a subsequent *H. suis* challenge compared to immunization with single proteins. A complete clearance was observed in approximately 50% of mice which is similar to the protection induced by a whole-cell lysate. This suggests that multiple antigens may be necessary for the development of a truly protective vaccine against *H. suis*. Combined vaccination with several protective antigens could result in a synergistic protective effect compared to vaccination with the single proteins. It is also possible that effective immunity against bacteria is achieved more likely by combining different antigens participating in different aspects of the pathogenesis of infection (Ferrero et al., 1995; Rossi et al., 2004). In any case, the observation that multiantigen vaccines induce better protection compared to one single antigen has been made for *H. pylori*, and other bacteria as well (Ferrero et al., 1995; Wu et al., 2008; Sadilkova et al., 2012).

An antigen should meet two main requirements to be considered an effective vaccine candidate (Blanchard and Nedrud, 2010). First, the protein should be present and conserved in all strains of the targeted *Helicobacter* species. Second, the antigen should induce a strong protective immune response when delivered as vaccine antigen with an appropriate adjuvant. To estimate how conserved an antigen is among different *H. suis* strains, proteins derived from many *H. suis* isolates should be tested in the future. In *H. pylori*, urease is well conserved and abundant, and is consequently the most commonly used antigen in *H. pylori* vaccination studies. Additionally, it has been shown that immunoreactivity to UreB is sustained across different *H. pylori* strains (Lock et al., 2002). The *H. suis* GGT encoding gene has been found in two sequenced *H. suis* strains (Chapter 1). However, it remains to be investigated how conserved this protein is among a larger *H. suis* population.

We demonstrated that vaccination with rUreB together with CT induced a strong humoral as well as local immune response in mice, which were both correlated with reduction of *H. suis* colonization, suggesting that rUreB is an effective antigen for vaccination against *H. suis*.

In contrast to the large number of studies using urease as vaccine candidate, only one study evaluated the protective efficacy of vaccination with the *H. pylori* GGT (Anderl et al., 2010). Both *H. pylori* and *H. suis* GGT inhibit T cell proliferation and thus interfere with the generation of an effective immune response (Schmees et al., 2007; Zhang et al., unpublished data). Mucosal immunization with *H. pylori* GGT in combination with the outer membrane protein, HpaA, induced a strong antibody response, and it was suggested that these antibodies block the enzymatic activity of GGT and thereby counteract its immunosuppressive effect (Anderl et al., 2010). Also in our study, we observed significantly higher anti-GGT antibodies in GGT-immunized and challenged mice compared to all other groups. Notably, immunization with rGGT alone did not induce strong gastric T cell responses, as expression level of Th1, Th2 and Th17 cytokines were not significantly different from that of non-immunized *H. suis*-infected animals. In addition, vaccination with rGGT alone elicited a limited protection against *H. suis* challenge compared to immunization with rUreB alone. Since GGT is a secreted protein, specific immune cells induced by the GGT-based subunit vaccine are likely to fail in targeting the pathogen. To overcome this, it has been suggested to vaccinate with GGT in combination with an outer membrane protein (Anderl et al., 2010). We suggest that rGGT could be a candidate for vaccination against *H. suis*, however most likely as a component of a multiantigenic vaccine, e.g. in combination with UreB, HpaA or GroEL.

One of the most important progresses in vaccine development against *H. pylori* is the improved understanding of mechanisms of protection. Before the start of this PhD research, very little was known about the mechanism of vaccine-induced protection against *H. suis* infections. The studies described in Chapters 2 and 3 demonstrated that probably a combination of local Th1 and Th17 responses, complemented by antibody responses play a role in the protective immunity against *H. suis* infections. We observed that reduction of gastric *H. suis* colonization was significantly correlated with an increase of antigen-specific serum antibodies, indicating that circulating antibodies could play a role in protection against *H. suis*. These findings correspond with some *H. pylori* vaccination experiments where a strong relation between protection and the presence of antigen-specific antibodies, both humoral and local, has been described (Czinn et al., 1993; Goto

et al., 1999; Jeremy et al., 2006; Nyström et al., 2006; Nyström and Svennerholm, 2007; Morihara et al., 2007). Nevertheless, several other authors have been able to induce a similar level of protection in antibody-deficient mice compared to wild-type animals, indicating that antibodies are not essential for protection against *H. pylori* (Blanchard et al., 1999; Gottwein et al., 2001; Garhart et al., 2003). Future research should elucidate which role humoral and/or local antibodies play in vaccine-mediated immunity against *H. suis*.

In our studies, an increased gastric response of both Th1 and Th17 (measured by their signature cytokines, IFN- γ and IL-17) was significantly correlated with higher levels of protection. Several studies showed a positive correlation between IFN- γ production by CD4⁺ T cells and protection in mice after immunization with *H. pylori* lysate (Akhiani et al., 2002; Rahn et al., 2004; Sayi et al., 2009). Also IL-17 has been suggested as a considerable cytokine in vaccine-mediated protection against *H. pylori* (DeLyria et al., 2009; Velin et al., 2009; Flach et al., 2011). Experimental infection of mice with pure cultures of *H. suis* leads to a predominant Th17 response and a secondary Th2 response (Flahou et al., 2012). Despite this response, *H. suis* persists in the stomach. Our vaccination studies indicate that for a significant protection a combination of Th17 and Th1 responses in the stomach is required.

In addition, we found that reduction of bacterial colonization after vaccination was significantly correlated with decreased expression levels of IL-10, an anti-inflammatory cytokine. Regulatory T cells (Tregs) are capable of producing large amounts of IL-10 (Roncarolo et al., 2006) and are currently considered as a major obstacle for successful vaccination against *Helicobacter* infections. Indeed, Tregs activated in the gastric mucosa have a negative effect on dendritic cells, Th1 and Th17 responses (Kao et al., 2010), and thereby hinder the development of an effective cell-mediated immune response against *H. pylori*, and most probably also against *H. suis*. A successful *H. suis* vaccine must therefore be able to tip the immune system balance in favor of Th1 and Th17 responses that will overcome the immunosuppressive properties of Tregs and IL-10. To limit the negative effect of Tregs, Bayry and coworkers (2008) introduced the use of CCR4 antagonists as adjuvants. CCR4 is expressed by Tregs and is the receptor for CCL22 and CCL17, two chemotactic cytokines for Tregs *in vitro* and *in vivo* (Iellem et al., 2001). Blocking CCR4 function and thus inhibiting the immunosuppressive effect of Tregs at the time of vaccination has been suggested to enhance vaccine-induced immune responses and could be promising for further *H. pylori* and *H. suis* vaccine development (Davies et al., 2008).

The exact role of T cell subsets and their cytokines in vaccine-induced protection against *H. suis*, however, should be further investigated, possibly by using either CD4⁻ or CD25⁻ mice or animals deficient in the cytokine of interest (Matsumoto et al., 2005; Velin et al., 2009).

An effective vaccine should induce satisfactory protection against infection whilst limiting side-effects. In our studies, we observed an enhanced gastritis four weeks after *H. suis* challenge in animals vaccinated with whole-cell lysate compared to non-vaccinated *H. suis* infected mice. This effect is an important issue in murine prophylactic *H. pylori* immunization studies, and has been referred to as “post-immunization gastritis” (Goto et al., 1999; Garhart et al., 2002; Morihara et al., 2007). A hypothesis for the development of this post-immunization gastritis is that in an immunized host, challenge with a gastric *Helicobacter* will lead to an enhanced gastric inflammatory response which could be critical for elimination of the bacteria (Mohammadi et al., 1996; Blanchard and Nedrud, 2010). Indeed, the severity of this gastritis in *H. pylori* immunized/challenged mice is often inversely correlated with the degree of protection, an observation which was also made in our experiments (Chapters 2 and 3). Interestingly, we found that post-immunization gastritis was negligible in mice vaccinated with single proteins and mice immunized with combinations of rUreB and rGGT, although also in these groups a significant reduction and even clearance of *H. suis* was achieved. The reason for this different inflammatory, but similar protective outcome in animals immunized with subunit vaccines compared to lysate-immunized mice remains to be explored. In addition, post-immunization gastritis has been shown to be transient in both *H. pylori* and *H. felis* immunization experiments (Sutton et al., 2001; Garhart et al., 2002). For *H. suis*, it still needs to be investigated whether the disparity in gastritis between infected animals and immunized/challenged mice will also disappear over time.

It is clear that not only the antigen, but also the adjuvant, route of administration, vaccination protocol and mouse strain influence the outcome of vaccination. The protective efficacy of rUreB and rGGT in combination with another adjuvant and/or other administration route could probably result in different protective effects. Indeed, Flahou et al. (2009) showed that intranasal (mucosal) immunization with *H. suis* lysate and CT is more effective against a subsequent *H. suis* infection compared to subcutaneous (systemic) vaccination with a saponin-based adjuvant. In contrast, more recent work showed that subcutaneous vaccination with *H. suis* lysate and complete Freund’s adjuvant induces a similar reduction of gastric colonization compared to intranasal *H. suis* lysate plus CT immunization (Flahou B., personal communication, 2013). It remains

therefore to be elucidated if other delivery routes and/or adjuvants could improve the protective effect of vaccination with rUreB and rGGT.

Following successful vaccination studies in rodent models one could move to the target host, humans (in case of *H. pylori*) and/or pigs (in case of *H. suis*). It became apparent that *H. pylori* proteins, which were often shown to be highly protective in the *H. pylori* mouse model (Ermak et al., 1998; Cortesy et al., 2005; Harbour et al., 2008; Raghavan et al., 2010; Zhang et al., 2011), only conferred modest levels of protection against *H. pylori* infection in large animal hosts or in humans, irrespective of the route of antigen administration used (Dubois et al., 1998; Lee et al., 1999; Solnick et al., 2000; Kotloff et al., 2001; Aebischer et al., 2008). The cause of differences in vaccine efficacy between the models is unclear, but may be related to differences in the host or to bacterial differences (e.g. used strain). Host differences could be due to innate immune differences or differences in other factors including the age. A comparison of the murine and human immunoproteomes of *H. pylori*, however, revealed that the pattern of *H. pylori* proteins that become exposed to the mouse immune system appears to be similar to that in human *H. pylori* infections, suggesting that the mouse infection model is suitable for preclinical screening of antigen candidates (Bumann et al., 2002). In case of *H. suis*, this still needs to be examined in translational studies from mice to pigs. For *H. pylori*, only two studies have been described in gnotobiotic piglets (Eaton and Krakowka, 1992; Eaton et al., 1998). Prophylactic vaccination of piglets with *H. pylori* sonicate did not prevent infection by subsequent challenge but reduced bacterial colonization, whereas therapeutic vaccination was unsuccessful. One apparent difference between piglets and mice was the neutrophilic gastritis associated with vaccination of piglets, in contrast to a more lymphoplasmacytic post-immunization gastritis observed in mice (Eaton et al., 1998). Future research will point out whether this post-immunization gastritis and the residual bacteria in vaccinated piglets disappear after time, as observed in the mouse model (Garhart et al., 2002).

For the most part, immune cell populations identified in mice are also present in pigs. Globally functional orthologs for all cytokines involved in Th1/Th2/Th17/Treg and corresponding cells have been described in pigs as well (Murtaugh et al., 2009; Kiros et al., 2011; Käser et al., 2012). The results described in Chapters 2 and 3 are therefore promising regarding the development of an effective vaccine against *H. suis* infections in pigs. It remains however to be determined whether vaccine-induced protection can really protect against the persistent colonization with *H.*

suis and the subsequent development of gastric lesions. Many antibacterial vaccines, currently used in swine, induce only partial protection (Haesebrouck et al., 2004). Nevertheless, even if prophylactic vaccination can not prevent a *H. suis* infection, the decreased colonization due to immunization may be economically attractive, as seen for commercial vaccines against *Mycoplasma hyopneumoniae* (Maes et al., 2008) and *Actinobacillus pleuropneumoniae* (Sadilkova et al., 2012). Experimental *H. suis* infections indeed induce a 10% decrease in daily growth rate in pigs over an average period of 5 weeks (De Bruyne et al., 2012). In addition, vaccination of pigs against *H. suis* may help to protect humans against infections with this agent, even if there would be no effect on pig performance. Vaccination in production animals to protect humans from zoonotic disease, as done already for *Salmonella* Enteritidis in laying hens, is the perfect illustration of the “one health concept” (Cogan and Humphrey, 2003).

3. New insights into the antimicrobial susceptibility of *H. suis* isolates

Combination therapy with a proton pump inhibitor and antimicrobial agents (usually clarithromycin and amoxicillin and/or metronidazole) is effective to eradicate *H. pylori* in the majority of infected individuals (Collins et al., 2006). However, this multidrug regimen does not prevent re-infection and antimicrobial-resistant strains are increasingly common (Mégraud et al., 2013). In general, human patients suffering from severe gastric pathologies associated with NHPH infections are treated with the same therapeutic regimens as used for *H. pylori* (Morgner et al., 2000). Using a mouse model, it has been shown that *H. suis* infections can be treated with a combination of amoxicillin and omeprazole. However, differences in sensitivity were seen between different *H. suis* isolates, which might indicate acquired antimicrobial resistance (Hellemans et al., 2005). Since no other data on intrinsic and acquired resistance were available for *H. suis* when we started our studies, *in vitro* susceptibility of an available collection of *H. suis* isolates was determined (**Chapter 4**).

Considering the demanding growth requirements of this *Helicobacter* species, the agar dilution method, recommended by the CLSI to test antimicrobial susceptibility of *H. pylori* (CLSI, 2010), was not suitable for *H. suis*. We developed a modified agar and broth dilution method consisting of a biphasic medium at pH 5. The results from reference strains *E. coli* and *S. aureus*, tested in *H. suis* susceptibility assay conditions, indicate that the low pH may influence activity of some

antimicrobial agents, including gentamicin, clarithromycin, tylosin and lincomycin. Indeed, it has been shown that minimal inhibitory concentrations (MICs) of antimicrobial agents can increase in more acid circumstances (Mégraud, 2010), indicating that the acidic pH of the stomach may affect the stability and optimal activity of antimicrobials. For this reason, it became mandatory to include a drug that diminishes gastric acid secretion, e.g. a proton pump inhibitor (Vergara et al., 2003). The effect of inclusion of proton pump inhibitors on the suppression of colonization of NHPH in the stomach remains to be investigated.

In vitro susceptibility testing is highly indicative to detect acquired resistance. Acquired resistance was only detected to enrofloxacin in one *H. suis* strain. This antimicrobial agent is commercially available for treatment of pigs, and is frequently used to control bacterial intestinal disease in piglets in Belgium (Callens et al., 2012). Levofloxacin, the relative of enrofloxacin approved for use in humans, is used to treat various bacterial infections, including *H. pylori* infections when a clarithromycin-based therapy fails due to bacterial resistance (Romano et al., 2010). Worldwide, the application of levofloxacin-containing regimens is limited due to the rapidly rising *H. pylori* levofloxacin resistance rate (Kim et al., 2005; Bogaerts et al., 2006; Chang et al., 2009; Mégraud et al., 2013). Quinolone resistance in *H. pylori* is mainly attributed to mutations in the quinolone-resistance determining region (QRDR) of gyrase A (*gyrA*) (Moore et al., 1995; Liou et al., 2011). A point mutation in the QRDR of *gyrA* was detected in the enrofloxacin-resistant strain of *H. suis*. Further studies are necessary to determine if this point mutation is responsible for the observed resistance. Such studies may include introduction of the mutation into susceptible strains by natural transformation and subsequent evaluation of their altered susceptibility to enrofloxacin (Moore et al., 1995; Pasquali et al., 2007). Alternatively, spontaneous enrofloxacin-resistant mutants may be produced by passing susceptible strains on media containing enrofloxacin. Less susceptible strains should then be examined for the presence of mutations in the *gyrA* gene (Wang et al., 2001).

To overcome quinolone resistance, new quinolones such as gemifloxacin have been developed recently. It has been shown that gemifloxacin has a 5-times lower MIC against levofloxacin-resistant *H. pylori* isolates and could partially overcome quinolone resistance caused by *gyrA* mutation (Chang et al., 2012), making this antibiotic a possible alternative for treatment of individuals infected with resistant *H. suis* and/or *H. pylori* strains.

In our study, MICs of ampicillin and metronidazole showed a monomodal distribution but mostly fell in a higher MIC range than described for susceptible strains of other gastric *Helicobacter* species (Loo et al., 1997; Van den Bulck et al., 2005b). This may indicate a reduced susceptibility of *H. suis* to ampicillin and metronidazole, compared to other gastric *Helicobacter* species. It remains to be confirmed if this has practical implications for treatment of *H. suis*-infected patients.

It should be kept in mind that the results of this study are based on a small number of *H. suis* isolates. Further studies, including more isolates are needed to obtain a representative view on *H. suis* acquired resistance. However, as mentioned before, *H. suis* is an extremely fastidious organism and its isolation is very laborious and time consuming. It indeed requires several weeks to obtain a single isolate from porcine stomachs.

We also need to consider the predictive value of *in vitro* testing for the clinical outcome of infection. Although we may conclude that most antimicrobial agents showed a good *in vitro* activity against *H. suis*, this does not ensure that *H. suis* will be eradicated from the stomach of patients treated with one of these antimicrobials. With regard to the discrepancy between *in vitro* and *in vivo* testing, one could refer to the behavior of *H. pylori* which is highly susceptible to antimicrobials *in vitro* while its eradication in patients requires aggressive triple or quadruple therapy (Ecclissato et al., 2002; Mégraud et al., 2013). This indicates that *in vitro* susceptibility testing could give an indication about the susceptibility of a microorganism to an antimicrobial agent, but does not guarantee that therapy with that agent will be successful. In general, a multidrug therapy including two or three antimicrobial agents and an acid suppressor is necessary for eradication of gastric *Helicobacter* species from the stomach.

4. General conclusions

The results of this thesis provide new insights into the molecular pathogenesis of *H. suis* infections. The genome annotation of *H. suis* presented in Chapter 1 shows that this microorganism lacks homologs for genes encoding the major *H. pylori* virulence factors (e.g. CagA, VacA, BabA, SabA). Nevertheless, we identified NapA, GGT, HtrA and FldA as potential virulence factors of *H. suis*. Future research, using *H. suis* mutants should clarify the precise role of presented genes in colonization and virulence of *H. suis*.

We provided new insights that may help in the development of an effective vaccine against *H. suis* infections. First, the immunoproteome of *H. suis* provided a list of candidate antigens which can be used in future vaccination experiments against this pathogen. Second, we showed that immunization with the *H. suis* UreB and GGT is a valuable alternative for whole-cell lysate to induce a protective immune response against *H. suis* infections. Future studies in pigs are needed to confirm the protective efficacy of these antigens.

In the present PhD thesis, we developed an *in vitro* assay to determine the acquired and intrinsic resistance of *H. suis* strains and found that acquired resistance to fluoroquinolones may be present in *H. suis* field isolates.

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Summary

Helicobacter suis is a Gram-negative, spiral-shaped bacterium that colonizes the stomach of the majority of slaughter pigs worldwide. An infection with this microorganism has been associated with erosive and ulcerative lesions in the non-glandular part of the porcine stomach and with chronic gastritis. A reduction in daily weight gain in experimentally infected pigs has been described, emphasizing the importance of *H. suis* infections for the pig industry. Furthermore, it is the most prevalent non-*H. pylori* *Helicobacter* species colonizing the stomach of humans suffering from gastric disease. In humans, *H. suis* has been associated with gastritis, peptic ulcers and mucosa-associated lymphoid tissue lymphomas of the stomach. Pigs are considered to be an important source of human infections. Either contact with pigs or the consumption of raw or undercooked contaminated pork are proposed as source of infection for humans.

H. pylori uses a variety of factors to persistently colonize the gastric mucosa and to induce gastric pathologies in humans. For a long time, the lack of pure *H. suis in vitro* isolates hampered the progress of research on this bacterium. At the start of this thesis, virtually nothing was known about the presence of genes involved in gastric pathogenicity of *H. suis* and in its specific adaptation to the gastric environment. Our research group was the first to successfully isolate *H. suis in vitro* in 2008. This created new opportunities to perform the present PhD studies which have the general aim to obtain better insights into the pathogenesis of *H. suis* infections and to develop control strategies against this bacterium.

Effective measures to control *H. suis* infections may not only decrease the number of pigs suffering from gastric disease, but are also important from a public health point of view. The prevalence of *H. suis* infections in adult pigs is very high, indicating that the immune response after natural infection does not result in eradication of the bacterium from the stomach. Vaccination with whole-cell lysate was shown to protect against a subsequent *H. suis* infection in a mouse model. However, it was not known which bacterial proteins are important for induction of protective immunity against *H. suis* infections. In addition, at the start of this thesis, no data were available on the antimicrobial susceptibility pattern of *H. suis*.

In **Chapter 1**, we describe the genome sequence of two *H. suis* strains: *H. suis* strain 1 (type strain = LMG 23995^T) and 5, both isolated from the gastric mucosa of swine. After performing a draft pyrosequencing assay and assemblage, the genome was annotated by cross-mapping with three well-investigated *H. pylori* strains. As some virulence factors may differ between both

species, *ab initio* annotations of the *H. suis* genome were performed as well. Comparison with the *H. pylori* genome revealed that all genes described to be essential for gastric colonization are present in the *H. suis* genome. Homologs of genes encoding the pro-inflammatory *H. pylori* neutrophil-activating protein (NapA), the apoptosis-inducing γ -glutamyl transpeptidase (GGT), and some type IV secretion systems were also identified in *H. suis*. *H. suis* also possesses several presumptive virulence-associated genes, including homologs for *mviN*, the *H. pylori* flavodoxin gene (*fldA*) and the *H. pylori* high-temperature requirement A gene (*htrA*). Sequences coding for outer membrane proteins involved in adhesion to gastric cells, such as *H. pylori* adhesin A (HpaA), HorB and some Hof members were present in *H. suis* as well. On the other hand, *H. suis* lacks homologs of several other *H. pylori* adhesion factors, including genes coding for the blood group antigen-binding adhesin BabA, the sialic acid-binding adhesin SabA and the adherence-associated lipoproteins AlpA and AlpB. It was concluded that although genes coding for some important virulence factors in *H. pylori*, such as the cytotoxin-associated protein A (CagA) and the vacuolating cytotoxin (VacA), were not detected in the *H. suis* genome, homologs of other genes associated with colonization and virulence of *H. pylori* and other bacteria were present.

Previous studies in mice showed that *H. suis* infection does not result in protective immunity, whereas immunization with *H. suis* whole-cell lysate (lysate) protects against a subsequent experimental infection. In **Chapter 2**, we described for the first time immunoproteomics of *H. suis*. Two-dimensional gel electrophoresis of total *H. suis* proteins was performed followed by immunoblotting with pooled sera from *H. suis*-infected mice or mice immunized with lysate. Little reactivity against *H. suis* proteins was observed in post-infection sera. Sera from lysate-immunized mice, however, showed immunoreactivity against a total of 19 protein spots which were identified using mass spectrometry (LC-MS/MS). The *H. suis* urease subunit B (UreB) showed most pronounced reactivity against sera from immunized mice and was not detected by sera from infected mice. Other identified proteins included *H. suis* chaperonin GroEL, urease subunit A, flagellin A and elongation factor G. Based on this analysis, the immunoreactive UreB was selected for further *in vivo* testing. As a control we included *H. suis* NapA, which has been previously described as a possible virulence factor but was not recognized by sera of mice immunized with whole-cell lysate, nor by sera from *H. suis*-infected mice. In a subsequent study, the protective efficacy of intranasal vaccination of BALB/c mice with the *H. suis* UreB and NapA, both recombinantly expressed in *E. coli* (rUreB and rNapA, respectively), was compared

with that of *H. suis* lysate. Cholera toxin was used as adjuvant. We found that immunization with the rUreB and lysate induced a significant reduction of gastric *H. suis* colonization compared to non-vaccinated and *H. suis*-infected controls. Immunization with rNapA had no significant protective effect. Reduction in gastric bacterial load was correlated with increased mRNA expression levels of IL-17, IFN- γ , and antigen specific serum IgG. It was concluded that rUreB could be a promising vaccine candidate for the use in vaccines against *H. suis* infections. Also, we suggested that probably a combination of local Th1 and Th17 responses, complemented by antibody responses play a role in the protective immunity against *H. suis* infections.

In **Chapter 3**, it was evaluated whether inclusion of an additional antigen could improve the protective efficacy of subunit vaccination in a mouse model. Mice were intranasally immunized with rUreB, *H. suis* GGT, recombinantly expressed in *E. coli* (rGGT) or a combination of both proteins, administered simultaneously or sequentially. Control groups consisted of non-immunized and non-challenged mice (negative controls), sham-immunized and *H. suis*-challenged mice (sham-immunized controls), and finally, *H. suis* lysate-immunized and *H. suis* challenged mice. Cholera toxin was used as mucosal adjuvant. All immunizations induced a significant reduction of gastric *H. suis* colonization, which was least pronounced in the groups immunized with rGGT and rUreB alone. Consecutive immunization with rGGT followed by rUreB and immunization with the bivalent vaccine improved the protective efficacy compared to immunization with single proteins, with a complete clearance of infection observed in 50% of the animals. Immunization with whole-cell lysate induced a similar reduction of gastric bacterial colonization compared to rGGT and rUreB in combinations. Gastric lesions, however, were less pronounced in mice immunized with combinations of rUreB and rGGT compared to mice immunized with whole-cell lysate. In conclusion, vaccination with a combination of rGGT and rUreB protected mice against a subsequent *H. suis* infection and was not associated with severe post-vaccination gastric inflammation, indicating that it may be a promising method for control of *H. suis* infections.

In vitro antimicrobial susceptibility of *H. suis* can not be determined using standard assays, as this agent only grows in a biphasic medium with an acidic pH. Therefore, in **Chapter 4**, we developed a combined agar and broth dilution method to analyse the activity of nine antimicrobial agents (ampicillin, ceftiofur, clarithromycin, enrofloxacin, gentamicin, lincomycin,

metronidazole, tetracycline, and tylosine) against nine *H. suis* isolates. After 48h microaerobic incubation, minimal inhibitory concentrations (MICs) were determined by software-assisted calculation of bacterial growth. Only for enrofloxacin a clear bimodal distribution of MICs was demonstrated, indicating acquired resistance in one strain, which showed an AGT → AGG (Ser → Arg) substitution at codon 99 of *gyrA*. For the other antimicrobial agents a monomodal distribution of MIC values was observed, indicating absence of acquired resistance. For ampicillin, MICs were higher than those described for other gastric *Helicobacter* species, possibly indicating reduced susceptibility of *H. suis* to this antibiotic. For 7 isolates, MICs of metronidazole were also high. The significance of these findings for treatment of *H. suis* infections is not clear. It was concluded that the assay developed in this study is suitable for determination of the antimicrobial susceptibility of *H. suis* isolates, although activity of acid sensitive antimicrobial agents may be higher than predicted from MIC endpoints. As the results of this study are based on a small number of strains, additional tests are needed to determine if these results reflect the susceptibility of the *H. suis* population.

The present PhD studies confirm that *H. suis* plays a role in gastric pathology, as its genome contains genes known to be essential for gastric colonization as well as genes encoding proteins that probably play a role in the induction of gastric lesions. Our results represent the first steps in the development of an effective subunit vaccine against *H. suis* infections. Combined vaccination with the *H. suis* UreB and GGT is suggested as a promising vaccine strategy. Finally, we developed an *in vitro* assay to evaluate the intrinsic and acquired resistance of *H. suis* isolates, and showed that resistance to fluoroquinolones may occur in *H. suis* field strains.

SAMENVATTING

Helicobacter suis is een Gram-negatieve, spiraalvormige bacterie die wereldwijd frequent voorkomt bij varkens. Bij deze diersoort kan een infectie met dit micro-organisme chronische gastritis veroorzaken en de kiem wordt ook geassocieerd met erosieve en ulceratieve letsels in de pars oesophagea van de maag. Er werd aangetoond dat een experimentele infectie met *H. suis* bij varkens gepaard gaat met een daling in de dagelijkse gewichtsaanzet, wat het economisch belang van infecties met deze bacterie bevestigt. *H. suis* is ook de meest frequent voorkomende niet-*H. pylori* *Helicobacter* species bij mensen met maagklachten en wordt geassocieerd met chronische gastritis, maagulcera en lymfomen van het mucosa-geassocieerd lymfoïd weefsel. Er zijn duidelijke aanwijzingen dat varkens een bron van infectie kunnen zijn voor de mens, wat deze bacterie een zoönotisch belang geeft. Contact met varkens of de consumptie van rauw of onvoldoende verhit varkensvlees zijn een mogelijke besmettingsbron voor de mens.

Het gebrek aan zuivere *in vitro* culturen van *H. suis* belemmerde gedurende lange tijd de vooruitgang in het onderzoek op deze bacterie. Bij de aanvang van dit doctoraatsonderzoek was er dan ook zo goed als niets geweten over de aanwezigheid van genen die betrokken zijn in de pathogeniciteit van *H. suis*, noch over genen die belangrijk zijn voor de aanpassing van deze kiem aan de specifieke omgeving van de maag. *H. suis* werd in 2008 voor het eerst *in vitro* geïsoleerd, en dit aan onze onderzoeksgroep. Dit creëerde nieuwe mogelijkheden om een beter inzicht te verwerven in de pathogenese van *H. suis* infecties en voor de ontwikkeling van strategieën om deze infectie onder controle te krijgen bij de mens en het varken.

Doeltreffende maatregelen om *H. suis* infecties bij varkens te bestrijden, zullen niet enkel het aantal varkens dat lijdt aan maagpathologieën doen dalen, maar zijn ook van belang voor de volksgezondheid. De prevalentie van *H. suis* infecties bij volwassen varkens is zeer hoog, wat erop wijst dat de immuunrespons na een natuurlijke infectie niet voldoende is om de bacterie te elimineren uit de maag. In een muismodel werd aangetoond dat vaccinatie met volledig kiemlysaat bescherming kan bieden tegen een daaropvolgende infectie met *H. suis*. Het is echter niet gekend welke bacteriële eiwitten van belang zijn voor de inductie van bescherming tegen *H. suis* infecties. Bovendien was er bij de aanvang van dit doctoraatsonderzoek geen informatie beschikbaar over de *in vitro* antimicrobiële gevoeligheid van *H. suis*.

In **hoofdstuk 1** beschrijven we de genoomsequentie van twee *H. suis* stammen: *H. suis* stam 1 (type stam = LMG 23995^T) en 5, beide geïsoleerd uit de maagmucosa van varkens. Nadat het

genoom werd gesequeneerd en geassembleerd in contigs, werd het geannoteerd door middel van vergelijking met drie gekende *H. pylori* stammen. *H. pylori* is de frequentst voorkomende gastrale *Helicobacter* species bij de mens. Het is ook de best bestudeerde *Helicobacter* species. Aangezien de genen die een rol spelen in de virulentie niet noodzakelijk identiek zijn voor deze beide *Helicobacter* species, werden er ook *ab initio* annotaties van het *H. suis* genoom uitgevoerd. De vergelijking met het *H. pylori* genoom toonde aan dat alle genen die essentieel zijn voor de kolonisatie van de maagmucosa ook aanwezig zijn in het *H. suis* genoom. Homologen van genen die coderen voor het pro-inflammatoir *H. pylori* neutrofiel-activerend eiwit (NapA), het apoptosis-inducerend γ -glutamyl transpeptidase (GGT) en enkele type IV secretiesystemen werden geïdentificeerd in *H. suis*. *H. suis* beschikt bijkomend over een aantal genen die vermoedelijk geassocieerd zijn met virulentie, inclusief homologen voor *mviN*, het *H. pylori* flavodoxine gen (*fldA*) en het *H. pylori* “high-temperature requirement A” gen (*htrA*). Sequenties die coderen voor buitenste membraan eiwitten welke betrokken zijn bij de adhesie van *H. pylori* aan maagcellen, waaronder het *H. pylori* adhesine A (HpaA), HorB en enkele leden van de Hof familie eiwitten, werden eveneens in *H. suis* gedetecteerd. Anderzijds mist *H. suis* homologen van verschillende andere *H. pylori* adhesines, waaronder genen die coderen voor het bloedgroep antigen bindende adhesine BabA, het siaalzuur bindende adhesine SabA, en de met adhesie geassocieerde lipoproteïnes AlpA en AlpB. Uit dit onderzoek werd geconcludeerd dat in het *H. suis* genoom genen ontbreken die coderen voor verschillende virulentiefactoren die een belangrijke rol spelen in de pathogenese van *H. pylori* infecties, zoals het cytotoxine geassocieerde eiwit A (CagA) en het toxine verantwoordelijk voor celvacuolisatie (VacA). Anderzijds werden homologen van een aantal andere genen die geassocieerd worden met kolonisatie en virulentie van *H. pylori* en andere bacteriën wel aangetoond in *H. suis*.

Vroeger onderzoek bij muizen toonde aan dat een infectie met *H. suis* niet leidt tot beschermende immuniteit, terwijl vaccinatie met volledig kiemlysaat (lysaat) van *H. suis* bescherming kan geven tegen een daaropvolgende experimentele infectie. In **hoofdstuk 2** werd voor het eerst het immunoproteoom van *H. suis* beschreven. Hiervoor werd tweedimensionele gelelektroforese van *H. suis* eiwitten uitgevoerd, gevolgd door Western blot analyse met gepoolde sera van *H. suis*-geïnfecteerde muizen of van muizen geïmmuniseerd met lymfaat. Sera van geïnfecteerde dieren toonden weinig reactiviteit tegenover *H. suis* eiwitten. Sera van muizen, gevaccineerd met lymfaat, toonden daarentegen een immunoreactiviteit tegen 19 eiwitspots, die vervolgens werden

geïdentificeerd met massaspectrometrie (LC-MS/MS). Sera van met lysaat geïmmuniseerde muizen reageerden sterk met het *H. suis* urease subunit B (UreB) terwijl sera van geïnfecteerde dieren dit eiwit niet detecteerden. Andere eiwitten die herkend werden door sera van met lysaat geïmmuniseerde muizen waren het *H. suis* chaperonine GroEL, urease subunit A, flageline A en elongatie factor G. Gebaseerd op deze analyses werd het immunoreactieve UreB geselecteerd voor verdere *in vivo* testen. Als controle werd het *H. suis* NapA opgenomen. *H. suis* NapA werd eerder beschreven als een mogelijke virulentiefactor van *H. suis*, maar werd noch gedetecteerd door sera van met lysaat geïmmuniseerde muizen, noch door sera van *H. suis*-geïnfecteerde muizen. In een daaropvolgende studie werd bij BALB/c muizen het beschermend vermogen nagegaan van een tweemaalige (3 weken interval) intranasale vaccinatie met het *H. suis* UreB en NapA, beide recombinant tot expressie gebracht in *E. coli* (respectievelijk rUreB en rNapA). Dit werd vergeleken met het beschermend vermogen van *H. suis* lysaat. Cholera toxine werd aan alle vaccins toegevoegd als adjuvans. De dieren werden experimenteel besmet met *H. suis* op 4 weken na de laatste vaccinatie. Niet gevaccineerde muizen fungeerden als controledieren. Bij muizen die geïmmuniseerd werden met rUreB en lysaat was het aantal *H. suis* bacteriën in de maag significant lager in vergelijking met geïnfecteerde controledieren. Immunisatie met rNapA had daarentegen geen significant beschermend effect. De verminderde bacteriële kolonisatie in de maag was gecorreleerd met verhoogde mRNA expressie niveaus van IL-17 en IFN- γ in de maag en met verhoogde antigen-specifieke serum IgG. Er werd geconcludeerd dat rUreB een veelbelovende kandidaat is om in te sluiten in vaccins tegen *H. suis* infecties. Tevens werd gesuggereerd dat een combinatie van lokale Th1 en Th17 reacties, aangevuld met opbouw van antistoffen, een rol spelen bij de bescherming tegen *H. suis* infecties.

In **hoofdstuk 3** werd in het muismodel onderzocht of toevoeging van een extra antigen het beschermend vermogen van subunit vaccins kan verbeteren. Muizen werden hiervoor intranasaal geïmmuniseerd met rUreB, *H. suis* GGT dat recombinant tot expressie werd gebracht in *E. coli* (rGGT), of met een combinatie van beide eiwitten, gelijktijdig of achtereenvolgens toegediend. De controlegroepen bestonden uit: i) niet-geïmmuniseerde/niet-geïnfecteerde muizen (negatieve controles), ii) muizen geïnfecteerd met *H. suis* na voorafgaande intranasale toediening van een steriele fysiologische zoutoplossing (niet-geïmmuniseerde, maar geïnfecteerde controles) en iii) muizen geïmmuniseerd met *H. suis* lysaat en daaropvolgend geïnfecteerd met *H. suis*. Cholera toxine werd opnieuw gebruikt als mucosaal adjuvans. Alle immunisaties zorgden voor

een significante daling van het aantal *H. suis* bacteriën in de maag in vergelijking met de niet-geïmmuniseerde, maar geïnfecteerde controlegroep. Deze daling was het minst uitgesproken in de groepen die enkel geïmmuniseerd werden met rGGT of rUreB. Achtereenvolgende immunisatie met rGGT en rUreB en immunisatie met het bivalente vaccin verbeterde het beschermend vermogen ten opzichte van immunisatie met één enkel eiwit, waarbij bij 50% van de dieren *H. suis* zelfs niet meer in staat was om de maag te koloniseren (volledige bescherming). Immunisatie met volledig kiemlysaat induceerde een vergelijkbare daling van de bacteriële kolonisatie in de maag ten opzichte van immunisatie met combinaties van rGGT en rUreB. Maagletsels waren echter minder uitgesproken bij muizen geïmmuniseerd met combinaties van rGGT en rUreB in vergelijking met dieren geïmmuniseerd met lysaat. Er werd besloten dat vaccinatie van muizen met een combinatie van rGGT en rUreB beschermt tegen een daaropvolgende *H. suis* infectie en dat deze immunisatiestrategie niet gepaard gaat met erge post-vaccinatie gastritis. Dit duidt erop dat het een veelbelovende methode kan zijn voor de bestrijding van *H. suis* infecties.

Aangezien *H. suis* enkel groeit in een bifasisch medium met lage pH, kan de *in vitro* antimicrobiële gevoeligheid van deze kiem niet worden bepaald door gebruik te maken van standaardmethodes. Daarom werd in **hoofdstuk 4** een gecombineerde agar en bouillon dilutie methode ontwikkeld. Deze werd gebruikt om de activiteit van 9 antimicrobiële middelen (ampicilline, ceftiofur, clarithromycine, enrofloxacin, gentamicine, lincomycine, metronidazole, tetracycline en tylosine) tegen negen *H. suis* isolaten te testen. Na incubatie van deze isolaten met tweevoudige verdunningen van de antimicrobiële middelen in een micro-aërobe atmosfeer gedurende 48 uur, werden de minimale inhibitorische concentraties (MICs) bepaald. Als MIC waarde werd die verdunning van het antimicrobiële middel genomen waarbij de bacteriële groei voor 50% geremd was. Dit werd bepaald met behulp van een *H. suis*-specifieke kwantitatieve PCR (qPCR). Enkel voor enrofloxacin werd een duidelijke bimodale distributie van de MICs aangetoond, wat wijst op verworven resistentie in één stam. Deze stam toonde een AGT → AGG (Ser → Arg) substitutie op codon 99 van het gyrase A gen (*gyrA*). Voor alle andere antimicrobiële middelen werd een monomodale distributie van de MICs waargenomen. Dit wijst op afwezigheid van verworven resistentie tegenover deze middelen in de hier geteste *H. suis* isolaten. De MICs van ampicilline lagen bij de *H. suis* isolaten hoger dan wat beschreven werd voor andere gastrale *Helicobacter* species, wat kan duiden op een lagere gevoeligheid van *H. suis*

voor dit antibioticum. Voor 7 *H. suis* isolaten lag de MIC van metronidazole ook hoog. De betekenis van deze bevindingen met betrekking tot behandeling van *H. suis* infecties, is onduidelijk. De methode die in deze studie werd ontwikkeld is bruikbaar voor de bepaling van de antimicrobiële gevoeligheid van *H. suis* isolaten, hoewel de activiteit van zuurgevoelige antimicrobiële middelen hoger kan liggen dan voorspeld op basis van de MIC eindpunten. Aangezien de resultaten van deze studie gebaseerd zijn op een klein aantal stammen, is aanvullend onderzoek nodig om te bepalen of deze resultaten de gevoeligheid van de *H. suis* populatie bij varkens weerspiegelen.

De resultaten van dit doctoraatsonderzoek toonden aan dat meerdere genen die essentieel zijn voor kolonisatie van de maag, aanwezig zijn in *H. suis*, evenals genen die coderen voor eiwitten die waarschijnlijk een rol spelen in de inductie van maagletsels. Onze resultaten vormen ook de eerste stap in de ontwikkeling van een effectief subunit vaccin tegen *H. suis* infecties, waarbij een veelbelovende kandidaat een combinatie van het *H. suis* UreB en GGT is. Verder onderzoek is evenwel noodzakelijk om dit te bevestigen, onder andere door het uitvoeren van experimentele infecties bij gevaccineerde varkens. Tot slot werd een *in vitro* test op punt gesteld die toelaat om de intrinsieke en verworven antimicrobiële resistentie van *H. suis* isolaten *in vitro* te bepalen. Met deze test werd aangetoond dat verworven resistentie tegen fluoroquinolones kan voorkomen bij *H. suis* isolaten van varkens.

CURRICULUM VITAE

Miet Vermoote werd geboren op 1 augustus 1984 in Oostende. Na het beëindigen van haar middelbare studies Wetenschappen-Wiskunde aan de Katholieke Centrumscholen te Sint-Truiden, startte ze in 2002 de studies Diergeneeskunde aan de Universiteit Gent. In 2008 behaalde ze met grote onderscheiding het diploma van Dierenarts, optie onderzoek. Onmiddellijk daarna startte zij aan diezelfde universiteit een doctoraatsonderzoek aan de vakgroep Pathologie, Bacteriologie en Pluimveeziekten, waarbij ze in het kader van een Geconcentreerde Onderzoeksactie (GOA) voornamelijk onderzoek verrichtte naar de aanwezigheid van virulentiegenen in *Helicobacter suis*. In 2009 behaalde zij een specialisatiebeurs toegekend door het Agentschap voor Innovatie door Wetenschap en Technologie van Vlaanderen (IWT). Dit liet haar toe om gedurende vier jaar onderzoek uit te voeren naar beschermende antigenen en antimicrobiële gevoeligheid van *Helicobacter suis*. Dit onderzoek resulteerde in het huidige proefschrift.

Gedurende deze periode heeft zij ook gewerkt aan de doctoraatsopleiding in Life Sciences and Medicine van de Universiteit Gent, waarvan zij het getuigschrift behaalde in 2013.

Miet Vermoote is auteur en medeauteur van meerdere wetenschappelijke publicaties in internationale en nationale tijdschriften. Zij nam actief deel aan internationale congressen en was daar meermaals spreker.

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